STUDIES OF PHYTOHEMAGGLUTININ, THE LECTIN OF *PHASEOLUS VULGARIS*

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ABSTRACT

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Lectins are toxic heat-stable glycoproteins that depress the protein quality of Phaseolus vulgaris varieties. Studies were designed to address the issue of lectin stability under a variety of conditions.

Kidney beans were exposed to controlled soaking and thermal treatments. Analysis of the hemagglultinating activity (HA) indicated that the time required for one log cycle reduction in HA decreased by 50 minutes for each 5.6^{o}C increase in holding temperature. Cooked kidney beans were palatable and showed no detectable HA after six hours at 91^{o}C. At 82^{o}C, kidney beans were palatable after 11 hours but showed detectable HA up to 14 hours.

Phytohemagglutinin was purified by affinity chromatography and exposed to thermal, chemical and enzymatic treatments. At 70^{o}C, the activity of the purified lectin diminished according to the equation:

$$\% \text{ activity remaining} = 102 - 9.87 \text{ (hours)}$$

Exposure to pH 12.0 sodium hydroxide solution or 5 M urea were the most effective chemical treatments for reducing the HA of the purified lectin corresponding to 65 and 39 % reduction in activity, respectively. Treatment with proteases resulted in 88 - 98 % reductions in HA after

three hours. Treatment of the purified lectin with mannosidase resulted in a slight but significant ($P < 0.01$) reduction in HA but treatment with amylases and neuraminidase had no effect.

Whole beans and bean flours were processed in a commmercial extruder. The HA of extruded products generally decreased with increasing final product temperature and increased barrel pressure enhanced this effect. Soaking kidney beans at pH 12.0 resulted in more rapid inactivation of HA and tenderization of the beans than samples at pH 7.0. Beans soaked at pH 12.0 reached a palatable end point in only 60 % of the time required for control samples at pH 7.0.

Of the small seeded Phaseolus vulgaris varieties analyzed, the most promising candidates for breeding for low lectin levels were Nep 2, P766, Carioca, Ica-pijoa, Jalpatagua, Jamapa and Black Turtle Soup. The most promising lines for high digestibility were Carioca, Protop-pi, 8217-111-24 and Sanilac. Electrophoretic analysis was not an adequate tool for screening dry bean lines for HA or digestibility.

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INTRODUCTION

Legumes play an important role in the diet of man. They are frequently a significant source of protein in the diets of the emerging and lesser developed nations. The importance of food legumes has been emphasized by the Protein Calorie Advisory Group of the United Nations (1974) who stated:

> "The food legumes are important and economical sources of protein and calories as well as certain vitamins and minerals essential to human nutrition. However, the significant role they play in the diets of many developing countries appears to be limited by their scarcity, caused in great part by their present low yield, their consequent cost, and certain defects in their nutritional and food use qualities."

Even though legumes are an important dietary component in many countries, they have not been subjected to research programs as intensive as those devoted to the cereal grains. This is due primarily to the difference in yield between the cereals and legumes, and the increased profitability of cereal crop production for farmers.

Legumes are coming under increased scrutiny because their potential dietary impact is so great. One group that is interested in the role of food legumes in developing countries is the United States government. The U.S. government, through the Foreign Assistance Act, funds a number of coordinated research programs in agricultural development in Africa and Central and South America. Title XII of the Act, entitled "Famine Prevention and Freedom from Hunger," provides funding for a number of Collaborative Research Support Programs (CRSP's) that integrate several agriculturally important disciplines in an effort to investigate crop

production and utilization at every level from production through consumption. These disciplines include genetics, plant breeding, entomology, pathology, agronomics, nutrition, food science and sociology.

The mission of one of these CRSP's, the Dry Bean/Cowpea CRSP, is to:

> "...establish active and vigorous collaborative research efforts that will contribute to the alleviation of hunger and malnutrition in developing countries by improving the availability and utilization of these legumes." (Dry Bean / Cowpea CRSP, 1984 Annual Report, Part I, Technical Summary).

It is projected that this CRSP will directly benefit the rural and urban poor and small farm producers in developing countries by increasing the supply of low cost nutritious legumes.

The Dry Bean/Cowpea CRSP is composed of 18 separate projects. One of these projects, "Improved Biological Utilization and Availablity of Dry Beans," is concerned with improving the quality and quantity of Phaseolus vulgaris varieties in rural settings in Guatemala. This is truly a collaborative project with investigators from five institutions in the U.S. and the Instituto de Nutricion de Centro America y Panama (INCAP) in Guatemala. The four research areas that this CRSP project addresses are: 1). production, 2). handling and storage, 3). utilization and consumption, and 4). processing and food product development.

This dissertation focuses on the evaluation of Phytohemagglutinin (PHA) , the lectin of P. Vulgaris, in raw and prepared dry edible beans. A sensitive PHA assay method was developed and utilized in a series of studies conducted to characterize this antinutritional protein. The first two studies of this dissertation deal with the

development of a hemagglutinating activity assay and the effect of various agents on the stability of purified phytohemagglutinin. This directly relates to the project area of utilization and consumption. The final two studies focus on the bean processing technology and the evaluation of P. vulgaris breeding line germplasm components of this project. This project was performed in conjunction with and partially supported by the Dry Bean/Cowpea CRSP.

Finally, this dissertation is presented as a series of four independent research papers preceded by three common chapters. The common chapters are an introduction, a review of the literature and a compilation of the materials and methods used. Each of the research papers is written to conform to the standards and guidelines for publication in the Journal of Food Science.

REVIEW OF LITERATURE

Beans in the Diet of Man

Nutritional Importance

The importance of food legumes, especially in the diets of emerging nations, is well established. Legumes have played a significant role in human nutrition since their consumption was initiated 4000 years ago (Bressani and Elias, 1977; Gomez Brenes et al., 1975; Elias et al., 1976). Today, out of approximately 13,000 species of legumes, only about 20 are commonly consumed by man, and most of this consumption occurs in the nations of Central America, South America, Africa and Asia.

Protein. The most important role of legumes in the diet is as an inexpensive protein source. This is crucial in the developing countries as they are frequently the major source of high quality protein in the diet. The protein quality of legumes is inferior to animal proteins but that problem is obviated by their complimentarity with cereal grains. Methionine is always the first limiting amino acid in legumes (Carpenter, 1981), but they are endowed with relatively high lysine levels. This explains their historic association with cereal grains in adequate, plant-based diets. Because legumes are looked upon as a good source of protein in vegetable based diets, much effort has been spent in investigating the protein quality of legumes and legume/cereal blends. The protein efficiency ratio (PER) of raw and cooked legumes is

approximately 0 and 1.2, respectively (Rockland and Radke, 1981). Bressani and Elias (1977) demonstrated an optimization of the apparent PER of legume/corn blends and found the PER was highest when the ratio of legume to corn was 3:7 (weight:weight).

Carbohydrate. Although legumes are often considered primarily as a source of protein, carbohydratres are the major component of P. vulagaris (55 - 65 % on a dry weight basis (db)). Starch (45 - 60 %) and dietary fiber (15 %) are the major carbohydrate constituents, while sugars contribute only 5 to 8 % to the carbohydrate fraction (Reddy et al. 1984).

Sahasrabudhe et al. (1981) and Reddy et al. (1984) determined that the starches of P. vulgaris comprise approximately 53 % (db) of beans. They also reported that these starches contained approximately 30 % amylose with a range of 15 to 38 %. This agrees with results reported in an earlier study of the starch in several legumes (Schoch and Maywald, 1968). These authors reported that the starch content of the beans ranged from 55 - 70 % (db) and amylose was present at a level of about 30 % in the starch fraction.

Monosaccharides and some oligosaccharides are present in dry beans but these make a minor contribution to the total carbohydrate. Soluble sugars are present in the range of 5.5 to 8 % (Reddy et al., 1984) and those present in the greatest concentration are sucrose and stachyose with raffinose and verbascose also present. Raffinose and the raffinose containing oligosaccharides constitute from 31 - 76 % of the total sugar fraction in dry beans (Naivikul and D'Appolonia, 1978; Akpapunam and Markakis, 1979).

The indigestible residue of dry beans is an important component of

the total carbohydrate fraction and makes up from 8 to 9 % crude fiber and about 23 to 25 % indigestible residue. The most prevalent fiber components in P. vulgaris varieties are cellulose, hemicellulose and lignins, but pectins are also present. Asp and Johansson (1981) reported that dry beans contain approximately 13 % water-insoluble fiber and 11 % water-soluble components. In addition to the carbohydrate based indigestible fibers, dry beans contain indigestible proteins that contribute to the indigestible residue (Bressani and Elias, 1977; Wolzak et al., 1981a).

Fats. Dry P. vulgaris varieties contain only 1 - 4 % and generally less than 2 % (db) fat (Sathe et al., 1981a). The majority (63.3 %) of the fatty acids are unsaturated (Watt and Merrill, 1963).

Vitamins. In addition to supplying carbohydrates and proteins, dry beans are a significant source of vitamins especially folic Acid, thiamin and pyridoxine. Recently, Augustin et al. (1981) published the mean concentrations for a number of water soluble vitamins and the contribution a 175 g sample would make towards the U.S. Recommended Dietary Allowances (RDA) for a number of P. vulgaris varieties. They reported that the concentration of folic acid ranged from 0.171 to 0.579 mg % and a serving contained on average 33 % of the RDA for this nutrient. Thiamin concentration ranged from 0.86 to 1.14 mg % with a serving containing 30 % of the RDA. Mean values for pyridoxine (vitamin B_6) ranged from 0.336 to 0.636 mg % and a serving contained 11 % of the RDA. Dry beans only supply 0.136 to 0.266 mg % representing 6 % of the RDA of riboflavin. Finally, niacin provided from 1.16 to 2.68 mg % or 4 % of the RDA. The fat soluble vitamins are present in dry beans but only in trace amounts.

Minerals. Dry beans have a fairly high ash content. Hosfield and Uebersax (1980) reported the ash content for 34 varieties of P. vulgaris and the average of the samples was 3.94 %. Augustin et al. (1981) reported the mineral content of dry beans and the contribution a 175 g serving made towards the U.S. RDA of the following minerals. The overall mean value for phosphorous was 0.46 mg % and a cooked serving provided 26 % of the RDA. The mean value for magnesium was 0.20 mg % and a serving provided 26 % of the RDA. The mean value for copper ranged from 0.69 to 1.20 mg % with the average serving providing 21 % of the RDA. Dry beans are also a good source of iron, providing 3.83 to 7.55 mg % and 19 % of the RDA. Zinc is present at levels of 2.2 to 4.4 mg % and a serving provides 12 % of the RDA. The mean values for calcium ranged from 0.09 to 0.2 % with a serving providing 9 % of the RDA. In addition to these minerals that have established RDAs, dry beans also provide sodium and potassium. The sodium content of dry beans ranges from 4 to 17 mg % and the potassium content is 1.5 mg %.

Breeding to Improve Agronomic and Food Quality Characteristics

Although legumes are a crucial source of amino acids, especially lysine, in the lesser developed countries their production is far below that of the cereal grains. The most important reason for this is that cereals are much more productive than legumes. Hulse (1977) states that on average, yields of corn, rice and wheat are 2.8, 2.2 and 1.7 tons/hectare compared to a yield of 0.5 ton/hectare for legumes (excluding soybeans). Because of the difference in yields, legumes are less likely to provide a satisfactory economic return to farmers than the higher yielding cereal crops. This is especially evident in Asia where the production ratio of cereals to legumes had increased to 9:1 by

1977 (Hulse, 1977).

Since the dietary legume to cereal ratio for optimum protein quality should be approximately 1:2 (Bressani and Elias, 1977), it is readily apparent that legume production should be stimulated. The most effective means to stimulate increased bean production would be to develop higher yielding legume varieties that would be more economically attractive to the small farmers in the developing countries. In addition to improving yield, there are other areas demanding further study: 1). improved nutrient content, especially total protein and sulphur amino acids; 2). increased digestibility; 3). reduction in antinutritional factors such as enzyme inhibitors and lectins; and 4). reduction in raffinose containing saccharides that may contribute to flatus production.

Processing Opportunities for Improved Dry Bean Utilization

In the developed nations, beans are generally prepared by commercial food processing operations and consumed as canned beans in sauce. The majority of legumes in the lesser developed nations are grown for home consumption by small farmers. Typically, the dry beans are soaked for a few hours, cooked in an open container and consumed whole or as a mashed bean paste with a ceral grain or tuber.

Raw legumes are poorly digested, but adequate heat treatment improves the digestibility significantly (Gomez Brenes et al., 1975; Wolzak et al., 1981a, 1981b). However, in many parts of the world the thermal treatment that can be provided for bean preparation in the home setting is not sufficient to inactivate toxic lectins (Coffey et al., 1985) and is often just sufficient to heat and hydrate the beans. Gomez Brenes et al. (1975) reported that peak digestibility and Protein

Efficiency Ratios of dry P. vulgaris were obtained after soaking for 8 or 16 hours and cooking at 121°C for 10 to 30 minutes. Heating for longer than this resulted in lower protein quality and decreased available lysine.

The effect of gamma irradiation on the nutritional value of P. vulgaris was reported by Reddy et al. (1979). The authors found that despite decreasing the protein solubility, the digestibility in all cases increased. However, this irradiation was in combination with autoclaving for 10 minutes. Data on the effect of irradiation on bean protein digestibility in the absence of autoclaving is not presently available.

Dehulling is another processing option that would in some cases improve the nutritional character of dry beans. Tannins concentrate in the hulls of colored beans and dehulling would be an efficient way to remove or reduce their levels. Elias et al. (1979) showed the presence of tannins to reduce the digestibilty of cooked beans. This was also more recently shown by Aw and Swanson (1985). Dehulling is already an important bean processing operation for the production of dhal (dehulled Vigna mungo) in India.

Soaking in mediums other than water may provide the possibility of improved nutritive value. It is commonly known that lye-treated corn is nutritionally better than untreated corn. The high pH soak increases the availabilty of niacin and lysine, which is the first limiting amino acid in corn. Soaking beans in water is recommended as it reduces the time necessary for adequate cooking (Molina et al., 1976). Further, Gatfield (1980) reported that trypsin inhibitors were reduced by water soaking prior to cooking. In spite of these reports, the effect of high

pH soaking on protein quality of beans has not been reported. This may be an effective approach that will prove advantageous in dry bean preparation from a nutritional perspective.

Novel processing operations may allow improved utilization of dry beans. For centuries, the Asian cultures have manipulated soybeans to produce a variety of nutritious food products. Some important soybean based foods are tofu, natto, miso, tempeh and tamari. If dry beans could be prepared by methods such as protein curd production or fermentation, the quality and digestibility may be improved. Swanson and Raysid (1984) described the changes in protein quality in tempeh made from red beans (P. vulgaris L.) and corn. There was a significant increase in digestibility and PER in the fermented products.

Thermal extrusion is another method of processing that has not been widely used in bean processing. High pressure extrusion would be beneficial if the pre-cooked bean products were nutritionally superior to dry beans. It would be expected that the digestibility and protein quality would improve with the heat treatment from extrusion but at this time it is not economically feasible in traditional settings.

Milling and fractionation of legumes into flours has recently received increased research effort. Bean flour production would permit the increased use of bean flour fractions in processed food products. Many high starch and high protein bean flour fractions have been produced, studied and incorporated into food products (Bakker-Akema et al., 1967; Schoch and Maywald, 1968; Sathe and Salunke, 1981a; Chang and Satterlee, 1979; Lee et al., 1985). These products are nutritionally desirable and have good functional atttributes in selected applications.

Lectins

Definition of Lectins

Boyd and Shapleigh (1954) reported that 57 members of the family Leguminoseae contained blood group specific antibody-like activity. Further, these authors found that protein extracts of Lima beans selectively agglutinated human type A erythrocytes and proposed the existence of a group of plant proteins with the ability to distinguish different blood types. The term "Lectin" (from the Latin Legere, to select or pick out) was selected to describe these proteins.

The term lectin has since been used in a generic manner to denote any protein with the ability to agglutinate erythrocytes. Unfortunately many proteins, such as toxins, immunoglobulins and some enzymes, have this ability under certain conditions. Agglutination of erythrocytes is therefore not sufficient to define lectins in a biological context. To further clarify the definition shortcoming, Goldstein et al. (1980) proposed that,

> "A lectin is a sugar binding protein or glycoprotein of non-immune origin which agglutinates cells and/or precipitates glycoconjugates."

They also stated that lectins should: 1). bear at least two sugar binding sites; 2). agglutinate animal or plant cells or precipitate polysaccharides, glycoproteins, and glycolipids; 3). be defined in terms of its carbohydrate inhibitor; 4). be soluble in biological fluids, or membrane bound.

These requirements would prohibit certain proteins such as carbohydrate-binding immunoglobulins and enzymes from the lectin category as they can be included in other structural and functional

categories. In a recent review, Barondes (1981) defined lectins in the following way:

> Lectins are "...divalent or multivalent carbohydrate-binding proteins that are grouped together because they agglutinate cells or other materials that display more than one saccharide of sufficient complementarity."

It appears that as our knowledge of lectins accumulates, an underlying functional characteristic of these proteins emerges. That is, all lectins interact with cell surface components or substances in the immediate vicinity of the surface and these interactions are usually due to the carbohydrate-binding ability of the proteins.

Function of Lectins

The metabolic role of lectins is yet unclear, but all the hypotheses that have been proposed for lectins are based upon their carbohydrate-binding abilities. The theories of greatest merit propose that lectins are involved in nodulation of legumes, protection of seeds against pathogens and predators, cell division and differentiation, and cell wall metabolism. A popular and logical hypothesis maintains that lectins play a role in bacterial recognition or binding during the root nodulation of legumes by Rhizobium bacterial species (Dazzo, 1981; Dazzo and Truchet, 1983; Pueppke et al., 1980; Pueppke, 1983; Pull et al., 1978; Law and Strijdom, 1984). The evidence for this role comes mainly from screening studies where several strains of nodulating or non-nodulating species of Rhizobium japonicum were exposed to soybean lectin. Bohlool and Schmidt (1974) found that 22 of 25 nodulating strains of R. japonicum bound a fluorescent derivative of soybean lectin. In contrast, none of the 23 non-nodulating strains bound the lectin derivative.

In general, Bohlool and Schmidt's results (1974) support the

hypothesis, however, there is also data against it. Pull et al. (1978) screened 102 lines of soybeans and found 5 which had no dectable lectin but could be nodulated by R. japonicum. Also, Pueppke et al. (1980) showed that there was no detectable lectin in soybean plants two weeks after germination. One problem advanced by the authors, however, was the possible existence of lectin in the root hair tips that may not have been extracted by their procedures.

In direct contrast to the results of Pueppke et al. (1980), and in fact illuminating the doubts about their procedure, are the results of Bowles et al. (1979). Performing a similar experiment but with an extraction using a detergent and sonication, the authors found considerable lectin activity in the roots of two week old soybean plants.

In addition to the work involving soybeans, there is a considerable body of evidence that supports a role for clover lectin in the mediation of binding the nodulating bacteria, Rhizobium trifolii. Dazzo and Truchet (1983) have recently reviewed the pertinent literature concerning lectin-receptor interactions in clover seedlings. Their presentation makes a convincing case for a physiological role for lectins in nodulation of clover by R. trifolii. Briefly, these data indicate that there is a host-specific mechanism in which lectin secreted from an actively growing root hair tip attaches to lectin receptors on the surfaces of the root hair tip and the Rhizobium, connecting the two.

An antibody-like role, in which lectins inhibit soil microorganisms, was advanced by Punin (cited in Jones, 1964). In support of this theory, Jones (1964), using Vicia cracca lectin,

inhibited the growth of a bacteria known to metabolize the seed coat of V. cracca. Further, Mirelman et al. (1975) showed that wheat germ agglutinin bound to the hyphal tips of Trichoderma viride and Fusarium solani, and inhibited their growth and germination. These results indicate that in certain cases, lectins act as a protective agent, preventing the microbial degradation of the seed.

Finally, lectins might play a protective role for seed survival by acting as a toxin to seed consuming insects. Janzen et al. (1976) found that lectins from black beans (P. vulgaris L.) were lethal to the Bruchid beetle larva, whose normal diet is the hemagglutinin-free Cowpea. These researchers believed the major adaptive significance of the legume lectins was to protect the seed from consumption by insects.

Nutritive Value

Although studies of lectins provide information about basic biological and chemical phenomena, the overiding reason that lectins are studied in relation to nutritional quality is because of their toxicity to man. The consumption of legumes is important in much of the world, especially the lesser developed countries. In fact, in Central and South America, beans are the most important food, after corn, in most rural communities (Gomez Brenes et al., 1975).

It has long been recognized that raw legumes are either poorly utilized by the body or even poisonous, but this is a minor problem as beans are rather unpalatable when raw. However, problems have occurred with improperly prepared beans. Noah et al. (1980) reported that in England since 1976, there were eight outbreaks of human poisoning attributable to consumption of red kidney beans. In every incident, every person that ate raw beans became ill. The clinical symptoms were

acute nausea and vomiting followed by diarrhea with occasional abdominal pain. The incubation time was relatively short in all cases, from one to three hours, and recovery was always complete. The smallest recorded dose that caused poisoning was four to five beans. Another pertinent aspect of this poisoning is the relative thermal stability of the toxin during low temperature cooking. Although most of the cases resulted from consuming uncooked beans, in two separate cases the beans were cooked in crock-pot cookers for 3 to 5.5 hours and those consuming the beans still became ill.

The anti-nutritive effect of raw kidney beans and phytohemagglutinin (PHA) have been clearly demonstrated. It has been known for many years that animals consuming raw legumes show limited or impaired growth. During early feeding trials, it was demonstrated that feeding raw kidney or black beans was lethal to rats (Jaffe, 1960). These results have consistently been supported by subsequent investigations (Honavar et al., 1962; Rattray et al., 1974; Pusztai et al., 1975; Untawale and McGinnis, 1977; King et al., 1980a, 1980b). In one of the earlier toxicological studies in this area, Jaffe (1960) demonstrated the oral and parenteral toxicity of purified PHA. All mice fed PHA at a level of 75 or more mg/kg body weight resulted in death during the feeding protocol. This work also showed that incorporation of 0.5 % PHA in the diet reduced the weight gain and increased the mortality of the test mice. In fact, this was the first demonstration of the toxicity of purified PHA.

Further support of the lethality of raw kidney beans came from Honavar et al. (1962), who demonstrated the heat lability of the toxic factor. When raw kidney or black beans were fed to rats at a level of

0.5 % of an otherwise adequate diet, all died within 13 days. However, when the beans were soaked overnight and autoclaved, rat growth response was equivalent to a casien-fed control group. Finally, the relative toxicity of the purified black bean hemagglutinin (BBA) and PHA were compared. The results showed that at equal levels in the diet, PHA was significantly more toxic than BBA. Jaffe and Vega Lette (1968), believing that the toxicity of kidney beans was due to its poor amino acid profile, attempted to overcome it by supplementing with methionine or pre-digested casien. They found though that regardless of the chemical quality, the presence of active PHA in the diet was lethal to rats.

Pusztai et al. (1975) fractionated kidney bean proteins and fed the fractions as components in a casien based diet. They found that the incorporation of either albumins or globulins in rat diets caused a loss in weight and reduced the Net Protein Utilization (NPU) of the diet from 88 to 39 and 37, respectively. Again, the raw-bean fed animals experienced high mortality, with a mean survival of seven days. Although they showed a depression in the NPU with both albumin and globulin fractions, due to their methods of separation, PHA may have been present in both fractions.

Hewitt et al. (1973) published an early report on the effect of raw navy beans on germ-free and normal chicks. They found that when raw navy bean meal was consumed, germ-free (gnotobiotic) chicks experienced a body weight depression of 12 %. However, when conventional chicks consumed this diet, their body weight depression was 48 %. Also, all chicks on the raw bean diet had enlarged pancreases. The authors concluded that the presence of the gut microflora aggravated the growth

depressing effect of the raw bean diet in chicks.

Jayne-Williams and Burgess (1974) studied the effect of raw navy bean meal on normal and gnotobiotic Japanese quail. They found that feeding raw navy beans to germ free animals posed no health risk but there was a significant increase in the amount of liver infections and the death rate in normal birds. Feeding studies using raw kidney bean and casien-based diets were conducted on normal and gnotobiotic rats by Rattray et al. (1974). Their results showing that germ-free rats survived but normal rats succumbed when fed an irradiated but raw bean diet, supported the results of Hewitt et al. (1973). Untawale and McGinnis (1979) proposed that since germ-free chicks survived a raw kidney bean diet, the normal gut flora had a role in the toxic reaction. They fed normal chicks raw kidney bean diets with or without penicillin and found that penicillin reduced the mortality rate from 51 to 27 % two weeks into the study.

The work with germ-free animals indicated that the gut microflora was responsible, at least in part, for the manifestation of kidney bean toxicity. This hypothesis was first advanced by Jayne-Williams and Burgess (1974), who believed that the high incidence of liver degeneration in navy bean-fed Japanese quail was indicative of coliform endotoxemia.

Toxicity Effects

To elucidate a mechanism of PHA toxicity, King et al. (1980a, 1980b) investigated the effect of PHA on the intestinal epithelium. They found that diets containing raw kidney beans or purified PHA resulted in severe disruption of the microvilli in the small intestine. There was no disruption in the casein-fed animals. The

bean-fed animals seldom survived longer than 5 days and when they died, their abdomens were distended and sensitive to pressure. The pathological characteristics induced by this diet included a thin fragile intestine, pancreatic hypertrophy and atrophy of the thymus gland, liver and spleen. The histopathological changes included irregularly arranged, stunted, elongated and fragmented microvilli. Also, the cells within the microvilli were different from the controls: they possessed differential staining characteristics, abnormal granules and poorly stained vacuoles. Associated with the damaged villi were flocculent debris and large numbers of bacteria. These authors believed that the toxic effect of PHA was due to impaired digestive and absorptive processes as a result of the lectin-induced epithelial damage.

Wilson, et al. (1980) investigated the microbiological effects of the PHA-induced intestinal damage. They found that when rats were fed a raw kidney bean diet, there was a three fold increase in the intestinal coliform count. Control animals had an average population of 1.3×10^7 organisms/g; Bean-fed rats had an average count of 4×10^7 organisms/g, with two individuals as high as 2×10^8 cells/g. Further, the microflora of control rats consisted mainly of Gram positive rods and cocci whereas the flora of bean-fed animals was predominantly Gram negative rods. These authors proposed a three point explanation for the coliform overgrowth: 1). impairment by PHA of the immunological suppression of E. coli in the gut; 2). PHA-induced aggregation or elimination of competing Gram positive rods and cocci; and 3). PHA-enhanced adhesion of E. coli to the brush border membranes. Finally, because of the pathological organ changes, these authors proposed that

the death attributed to PHA was due to coliform endotoxemia, the coliform toxins apparently being absorbed, intact or after only partial digestion, through the damaged brush border membranes. Support for the absorption of whole or partially degraded proteins in kidney bean-fed animals was reported by Pusztai et al. (1981). These authors found lectin antibodies in the sera of bean-fed rats.

This is an interesting topic in lectin biology and this explanation of PHA toxicity deals with the lethality in a realistic manner. If the PHA toxicity mechanism was simply explained as binding to active absorptive sites, the PHA-fed rats should survive as long as animals on deficiency diets. This is clearly not the case. Based on the results showing severely disrupted microvilli and materials that react with anti-PHA antibodies in the blood of rats fed PHA, the absorption of PHA or microbial toxins seems a credible explanation of PHA toxicity and worthy of further study.

Physical and Chemical Properties of PHA

Amino Acid Composition. Basic to any study of proteins is amino acid composition and sequence, since these are the ultimate determinant of the protein's physical and chemical characteristics. The amino acid composition of PHA was first reported in 1965 and it differs slightly from some recent reports, probably due to the difference in purification techniques available then and now. Amino acid data is compiled in Table 1.

Jaffe and Hannig (1965) were the first to report the amino acid composition of PHA, which was one of the ten components purified by ammonium sulphate fractionation of kidney beans. This globulin fraction had a high hemagglutinating activity but was not as pure as the PHA

purified by affinity chromatography and this probably explains the discrepancies in the amino acid profile (see Table 1).

Table 1. Amino acid composition of PHA or its subunits as reported in selected studies (I - VII)

	I	II	III	IV	V	VI	VII
RESIDUE			Mole Residue/Mole Lectin				
LYS	48.7	43.6	41.3	36.5	55.6	46.0	49.6
HIS	9.7	17.0	8.3	9.8	13.6	11.7	---
ARG	33.7	50.0	37.2	34.4	21.9	28.2	18.0
ASP	143.8	159.5	136.7	149.0	145.0	147.0	154.0
THR	90.4	78.0	83.4	82.9	105.0	94.0	91.2
SER	109.6	113.6	103.5	101.0	103.0	102.0	92.0
GLU	72.0	87.2	68.9	59.7	58.8	59.2	60.4
PRO	51.0	33.5	34.6	30.4	39.3	34.8	45.6
GLY	103.9	27.9	78.5	69.8	60.9	65.4	71.6
ALA	71.9	38.4	60.4	56.7	66.2	61.4	69.2
CYS/2	5.4	0.6	3.2	0	0	0	0
VAL	71.3	77.5	85.1	76.2	75.1	75.6	79.2
MET	1.0	11.6	0	0	0	0	0
ILE	80.2	48.0	43.1	46.2	51.5	48.8	52.4
LEU	42.4	53.8	83.5	91.3	109.0	100.2	96.8
TYR	29.3	45.8	17.6	20.3	24.7	22.5	8.8
PHE	62.4	71.2	61.2	61.6	57.6	59.6	55.6
TRP	21.9	32.6	21.1	26.3	23.1	24.7	12.0

I = Phaseolotoxin A (Jaffe and Hannig, 1965)
II = Glycoprotein I (Pusztai, 1966)
III = L-PHAP (Allen et al.,1969)
IV = L4 PHA (Leavitt et al., 1977)
V = E4 PHA (Leavitt et al., 1977)
VI = (L4 + E4)/2 (Leavitt et al., 1977)
VII = PHA (Ohtani et al., 1980)
CYS/2 = 1/2 Cysteine

Pusztai (1966) used high voltage electrophoresis and chromatography on Sephadex to purify PHA and Allen et al. (1969) also used a Sephadex column to purify commercial PHA. There is some discrepancy between the results of these two groups, particularly with the values for glycine, alanine, leucine, tyrosine, phenylalanine and tryptophan.

Recently, very pure samples of PHA have been produced by Leavitt et al. (1977) and Ohtani et al. (1980) using thyroglobulin-Sepharose and Concanavalin A-Sepharose affinity chromatography, respectively. The results of Ohtani and co-workers are slightly different from those of Levitt and his group. However, Ohtani determined the amino acids in the PHA eluted from the affinity column whereas Leavitt's group determined the compostion of each of five isolectins. The results of Ohtani's group did, however, correspond closely to the average of the values of the five isolectins with exception of histidine, glycine, tyrosine and tryptophan.

One interesting feature of the PHA is its lack of S-containing amino acids. One expects that given the low cysteine and methionine levels in legumes, the individual proteins would be low in these residues, but PHA has none. However, neither do Concanavalin A (Con A), the fava bean lectin or the lentil lectin (Foriers et al., 1981).

Amino Acid Sequence. The only extensive report on the amino acid sequence of PHA was published by Miller et al. (1973). These authors determined the identity of the first 24 residues of the lymphocyte-stimulating and erythroagglutinating isolectins. The first, 5th and the 7th residues differ in these two proteins, and residues six, and eight through 24 are identical. The sequence is as follows:

(A) SER -- ASN -- ASP -- ILE -- TYR -- PHE -- ASN --
1 3 5 7

(B) ALA -- SER -- GLN -- THR -- SER -- PHE -- SER --

(A+B) PHE -- GLN -- ARG -- PHE -- (?) -- GLU -- THR -- ASN --
9 11 13 15

LEU -- ILE -- LEU -- GLN -- ARG --ASP -- ALA -- SER -- VAL --
17 19 21 23

(A -- Strongest lymphocyte stimulator)

(B -- Strongest erythroagglutinator)

The authors suggested that the amino acid at position 12 may be glycosylated and this would explain why the Edman degradation did not identify this residue. They also proposed that it may be Asparagine as many other glycoproteins have glycosylated Asparagine residues separated by one amino acid from Threonine in the primary structure.

Subunit Composition. Rigas and Head (1969) were the first to probe the subunit structure of PHA. Using starch gel electrophoresis, PHA exposed to urea apparently separated into eight distinct bands. They tested each electrophoretically pure component for erythroagglutinating and lymphocyte stimulating activity and found that one of the bands was a potent lymphocyte stimulator and another was a strong erythroagglutinator. Based on their results, they proposed that PHA was composed of a total of eight subunits of only two types, an erythroagglutinating or a lymphocyte stimulating subunit.

Yachnin and Svenson (1972) also proposed that PHA was composed of two different subunits, but when they electrophoresed crude PHA, they found only five different protein bands in which the hemaagglutinating or lymphocyte stimulating activity was concentrated. Further, they found that dissociation of the protein with the highest lymphocyte-stimulating activity yielded four distinct subunits with a molecular weight of approximately 36,000, which had little or no hemagglutinating activity. From their results, they proposed that the PHA molecules are made up of various combinations of only two different subunits, one with a strong affinity for red blood cell membrane glycoproteins (R) and another a potent lymphcyte stimulator (L). They suggested that the most potent lymphocyte stimulating PHA was composed of four R subunits. The remaining three erthroagglutinating proteins were proposed to be made of the following combinations of subunits: 3 R and 1 L; 2 R and 2 L; and 1 R and 3 L.

Further support for this theory comes from Miller et al. (1973). This group separated the PHA into five proteins and determined the NH terminal amino acid residue. The strongest lymphocyte stimulator had serine and the strongest red blood cell agglutinator had alanine at the NH terminal position. They concluded that since their results showed there were four subunits per molecule, there must be five isomitogens of PHA with the same proportional composition proposed by Yachnin and Svenson (1972).

Leavitt and Felsted and their collegues have published several reports on the characteristics of PHA and its subunits. Leavitt et al. (1977) used thyroglobulin-Sepharose affinity chromatography to purify PHA which was further resolved into five distinct components by ion

exchange chromatography. Sedimentation studies showed a molecular weight of 115,000 ± 4,130 for each of the five species and when these were electrophoresed on SDS-polyacrylamide gel, all showed a single protein band with an apparent molecular weight of 33,000. These results strongly suggest that each isolectin contains four subunits.

In an extension of this work, Felsted et al. (1976) showed that the isolectins precipitated red blood cells and animal serum proteins in a stepwise manner. At this point, the authors proposed that there are only two different subunits, a strong erythroagglutinin (E) and a strong mitogen (L), and that the five isolectins are composed of various proportions of each. Therefore, the strongest erythroagglutinator was composed of four E subunits and the strongest mitogen was composed of four L subunits. The author named their five isolectins according to the number of E or L subunits each contained. They are E4, E3, E2, E1 and L4.

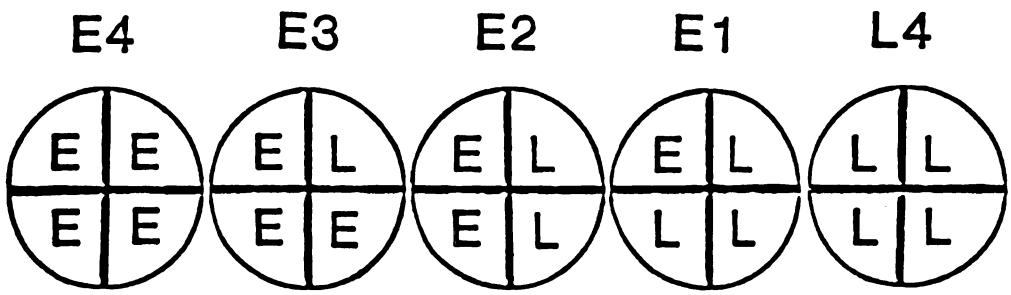
Felsted and his colleagues (1977) published a report concerning the recombination of dissociated isolectin subunits. Dissociation of E4 or L4, using 6M guanidine HCl followed by removal of the dissociating agent, yielded products that were indistinguishable from the original E4 and L4. Also, inclusion of labeled L4 into a dissociation reaction with E4 resulted in the formation of five distinct proteins that were electrophoretically equivalent to the native E4, E3, E2, E1 and L forms but had labeled L concentrations proportional to their proposed stuctures.

In a more sensitive study of the isolectins, Felsted et al. (1981) applied the five to continuous and discontinuous SDS-polyacrylamide gel electrophoresis. They found that as before, electrophoresis in a

continuous system resulted in only one major protein band with an apparent molecular weight of 33,000 for all isolectins. However, when subjected to a discontinuous system, two protein bands were resolved. The E4 species yielded only one component with a lower electrophoretic mobility than the one component of the L form. When these subunits were compared to molecular weight standards, the E and the L subunits had apparent molecular weights of 31,700 $\pm$ 600 and 29,900 $\pm$ 200, respectively. Also the hybrid isolectins (E3L1, E2L2 and E1L3) contained both protein subunits and were stained in relative proportion to their proposed subunit composition.

Based on the results of the subunit work by these groups, the proposed structures for the five isolectins are illustrated in Figure 1.

Figure 1. Proposed isolectin subunit composition



Molecular Weight. The estimates of the molecular weight of PHA range from a low of 115,000 to a high of 150,000. Allen et al. (1969) reported a molecular weight of 115,000 for the potent leucoagglutinin

fraction of PHA. This figure was derived from ultracentrifugation data.

Dahlgren et al. (1970) also used centrifugation data but came up with weights of 140,000 and 150,000 for the leucoagglutinin and erythroagglutinin, respectively. These authors also dissociated the purified PHA into subunits and electrophoresed them on SDS-polyacrylamide gel with molecular weight standards. They found that the subunits of the erythroagglutinin had an apparent molecular weight of 35,000 and those of the leucoagglutinin were 31,000.

Felsted et al. (1976) determined the weight of each of the five isolectins by ultracentrifugation and found them all to be 115,000 + 4,130 g/mole. The isolectins were also electrophoresed in SDS-PAGE and had apparent subunit weights of 33,000.

Pusztai and Palmer (1977) purified PHA on fetuin-Sepharose 4B and produced a 95 % pure PHA. The molecular weight, determined by molecular sieving on Bio-Gel A, was 120,000 and the subunit weight was 30,000 by SDS-PAG electrophoresis.

Ohtani et al. (1980) estimated the molecular weight of affinity purified PHA by exclusion chromatography on Sephadex G-200 and of the subunits by SDS-PAG electrophoresis. The native PHA was 130,000 g/mole and the subunits were 32,000 g/mole. Felsted et al. (1981) subjected the purified E4 and L4 isolectins to SDS-PAGE and found the apparent molecular weights to be 31,700 $\pm$ 600 and 29,900 $\pm$ 200, respectively.

In comparison to the lectins of red kidney beans, Junqueira and Sgarbieri (1981) showed that the molecular weight of the lectin of the pink bean (*P. vulgaris* var Rosinha G2) was 136,000 by chromatography on Sephadex G-200 and ranged from 136,000 to 160,000 by ultracentifugation.

Carbohydrate Composition. There is good agreement about the carbohydrate composition of PHA. Allen et al. (1969) reported that the carbohydrates of PHA were mannose (4.76 %), N-acetyl-D-glucosamine (2.3 %), Xylose (0.48 %) and arabinose or fucose (0.38 %). Oh and Conrad (1971), Pusztai and Watt (1974) and Junquiera and Sgarbieri (1981) reported glucosamine concentration of 2.0, 1.8, and 2.12 %, respectively. Ohtani et al. (1980) reported the results of a comprehensive fractionation of PHA carbohydrate and they found the total neutral sugars comprised 7.8 % of the PHA weight. Further, the sugars involved and their molar ratios were: mannose, N-acetyl-D-glucosamine, L-fucose and D-xylose (9.6:2.0:0.6:0.7). Based on the figures of Ohtani et al., and a molecular weight of 30,000 daltons for one PHA subunit, there would be approximately 13 carbohydrate moities per subunit. Ohtani et al. (1980) released five of nine moles of mannose by digestion with a mannosidase with no change in agglutinating activity. Lotan et al. (1975) showed on the basis of periodate oxidation that mannose was not responsible for the hemagglutinating activity.

Sedimentation Coefficient. Allen et al. (1969), Weber et al. (1972) and Ohtani et al. (1980) have published values for the sedimentation coefficient of PHA. Allen and co-workers reported a value of 6.5 for the L4 subunit. The E4 subunit was not analyzed. Weber et al. (1972) subjected both L and E PHA species to centrifugal analyis and they reported values of 6.8 and 7.1, respectively. Ohtani and co-workers (1980) analyzed affinity purified PHA by centrifuge and found an S of 5.6.

Partial Specific Volume. This characteristic of PHA has been determined in older studies but not in any current work with affinity purified PHA or isolectins. Allen et al. (1969) determined the V of L-PHAP (L4) to be 0.73. Weber et al. (1972) showed both the E and L forms of PHA to have identical values of 0.75. Similarly, Juqueira and Sgarbieri (1981) reported a value of 0.75 for pink bean lectin.

Frictional Ratio. Weber et al. (1972) are the only group to report the frictional ratio of PHA. The L and E forms had the same value of 1.3. This is also the value reported for the pink bean lectin (Junqueira and Sgarbieri, 1981).

Diffusion Coefficient. Allen and co-workers (1969) are the only group to report the diffusion coefficient of PHA. By sieving on Sephadex G-150 the L-PHAP (L4) had a D of 5.1 and the H-PHAP (E4) was 4.76. Junqueira and Sgarbieri (1981), using Sephadex G-200, reported a D of 5.0 for pink bean lectin. They also calculated the stoke's radius of the lectin from the chromatography data to be 43 angstoms.

Isoelectric Point. Weber et al. (1972) reported the pI of 5.0 for the leucoagglutinin (L4) and 6.5 for the erythroagglutinin (a mixture of E containing isolectins). However, Felsted et al. (1976) found that the purified E4 and L4 isolectins exhibited slight heterogenicity on isoelectric focusing in urea-containing polyacrylamide gels. The pI ranges for E4 and L4 were 5.6 - 5.7 and 5.4 - 5.6 respectively.

Felsted and co-workers (1976) explained the heterogeneity of the pI as due to differences in the amino acid composition of the subunits arising from genetic variation or post-ribosomal modification or both. They believed that the differences were not due to differences in the carbohydrate compositon as both E4 and L4 contain only uncharged mannose

and N-acetyl-D-glucosamine.

Binding Properties

Metal Binding. One of the earliest reports concerning the absolute requirement of metals for hemagglutinins of the Phaseolus genus was authored by Galbraith and Goldstein (1970). Lima bean lectin was demetalized by successive dialysis against EDTA and acetic acid. The authors showed that the metal-free lectin had only 25 % of the original activity, but this increased to 90 % when calcium, magnesium or manganese were added back to the metal-free protein.

Ohtani et al. (1980) are the only group to investigate PHA for a metal ion requirement. They found that by dialyzing PHA against 1 M acetic acid, the fetuin-precipitating ability of the protein decreased by 70 %. The fetuin-PHA precipitation was also inhibited by the addition of EDTA, although they did not quantify the loss of activity. The addition of calcium, manganese and magnesium to the metal-free PHA restored the fetuin precipitating activity to 100, 80 and 60 % of the original, respectively.

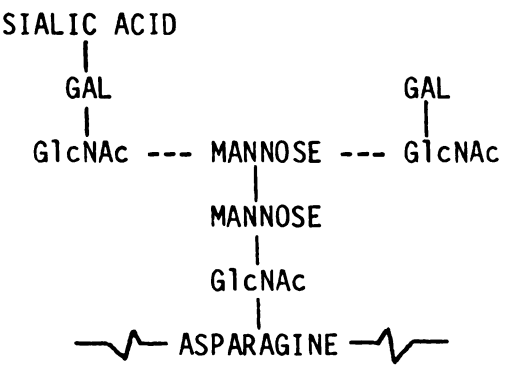
The ability of calcium to restore PHA activity is consistent with the results of metal-binding determinations of lima beans and soybean lectins and Con-A. These three lectins each have an absolute requirement for divalent cations, of which calcium seems most active.

Carbohydrate and Glycoprotein Binding. The characteristic of PHA and other lectins that is responsible for the scientific community's interest in it is its carbohydrate binding ability. This endows the lectin with potent biological activity and allows it to be used as a probe for cell surface characteristics. Many lectins are strongly inhibited by simple sugars, PHA is not. PHA binds to glycoproteins.

In 1966, Borberg and colleagues showed that N-acetyl-D-galactosamine selectively inhibited the PHA-induced agglutination of rat lymphocytes. Other similar carbohydrates, such as galactose, N-acetyl-D-glucosamine, glucose and fructose, had no significant effect on this reaction. Following this work, Borberg et al. (1968) reported that the agglutination of erythrocytes as well as lymphocytes was specifically and reversibly inhibited by 10-50 mg/ml of N-acetyl-D-galactosamine.

Although PHA does not bind strongly to simple sugars, it binds tenaciously to glycoproteins. In 1970, Kornfeld and Kornfeld isolated a soluble glycoprotein from human erythrocyte membranes that abolished PHA's erythrocyte agglutination and lymphocyte stimulating activity. This pure glycoprotein, isolated by trypsin treatment, had a molecular weight of approximatley 2,000 and the following composition: sialic acid, galactose, mannose, N-acetyl-D-glucosamine, aspartic acid, serine and threonine. Sequential cleavage of the carbohydrate residues led the authors to propose the following structure:

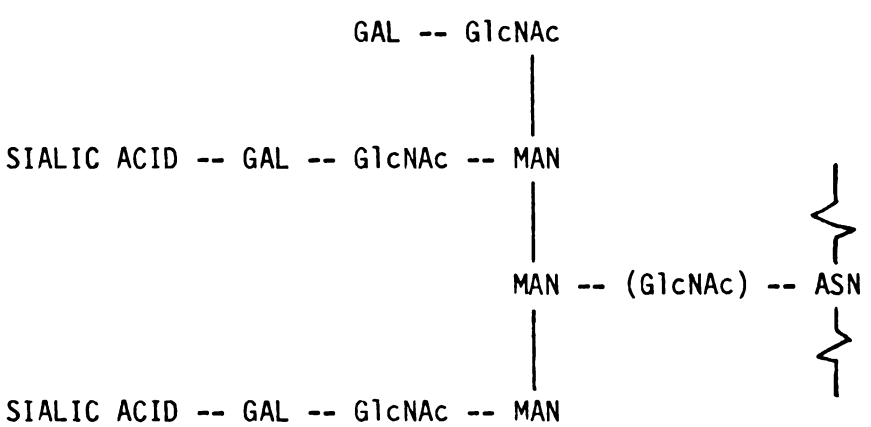
Figure 2. Human erythrocyte glycoprotein oligosaccharide



Cleavage of the sialic acid residue failed to affect activity, but treatment with α-galactosidase rendered the glycoprotein incapable of inhibiting PHA-induced erythrocyte agglutination. In addition to this, glycopeptides isolated from immunoglobulin G (Kornfeld et al., 1971) and fetuin (Spiro, 1964), which also have internal mannose residues and galactosylsialic acid sequences in branches, are also potent inhibitors of PHA activity.

Toyoshima and co-workers (1972) investigated the inhibition of labelled thymidine uptake by lymphocytes with or without glycoproteins, when exposed to various lectins. They found that each of the four lectins tested, all of which had different sugar specificities, were inhibited by porcine thyroglobulin glycopeptides. The proposed structure for the glycopeptide used by this group is shown in Figure 3.

Figure 3. Porcine thyroglobulin oligosaccharide



Irimura et al. (1975), using glycopeptides and glycosides of galactose and 2-acetamido-2-deoxy-galactose, determined the carbohydrate-binding specificity of lectins from the following

sources: Bauhinia purpurea, Ricinus communis, Maackia amurensis, Sophora japonica, Wistaria floribunda, soybean, peanut and red kidney bean. They reported that the lectins could be divided into three classes based on the type of sugar chain they preferentially react with. PHA, R. communis lectin and M. amurensis lectin were strongly inhibited by porcine thyroglobulin glycopeptide B (see Figure 3). The other lectins failed to react with this glycoprotein but were inhibited by simpler unbranched oligosaccharides. From these studies, it seems that two things are required of the carbohydrate portion of a glycoprotein for binding to PHA: 1). a branch containing at least one galactose residue prior to a terminal sialic acid and 2). a poly-mannose region in the carbohydrate core.

Although it is convenient to categorize the lectins based on their binding specificities for simple sugars, they do have higher affinities for complex carbohydrate structures (Pereira and Kabat, 1979). Reasoning that lectins most likely interact with complex cell-surface components in their native state, Young and colleagues (1982) used NMR to study the structural changes induced in lectins by binding different carbohydrates. They found that binding glycopeptides resulted in less comformational change than when binding small saccharides. This fact, coupled with lectins' higher affinities for glycoproteins than simple sugars, led the authors to propose that the binding sites on lectins are preformed for appropriate glycoproteins. They also maintained that based on the conformational results, interactions of lectins with glycopeptides is probably more relevant to their in vivo activity than is their behavior with simple sugars. Much still remains to be learned about the nature of lectin-carbohydrate binding, but it seems logical

that the interaction with oligosaccharides and glycoproteins most closely resembles the PHA-induced events at the cell surface.

Analytical Methods

Fractionation. Early work with PHA purification involved ammonium sulphate precipitation and dialysis (Rigas and Osgood, 1955; Jaffe and Gaede, 1959; Jaffe, 1960). The main drawbacks to these procedures were the low yield and suspect purity.

Pusztai (1966) purified the protein by ammonium sulphate fractionation and dialysis followed by high voltage electrophoresis and chromatograghy on a number of Sephadex columns. He found that one of the fractions, homogenous by electrophoresis, could be resolved on Sephadex G-200, G-75 and DEAE columns into a glcoprotein (PHA) and a trypsin inhibtor.

Oh and Conrad (1971) described the use of preparative disc gel elecrophoresis to isolate the mitogenic components of a commercially available PHA. They used a separating gel of 7-8 % polyacrylamide containing 4M urea at pH 9.0 and a stacking gel containing 4M urea at pH 6.8. The mitogenic components were then eluted with 0.05 M Tris-0.4 M glycine buffer. Using this procedure, they were able to isolate 2 mitogenic fractions from the PHA.

Affinity Chromatography. In the late 60's and early 70's, affinity chromatography became the preferred method for lectin purification. Matsumoto and Osawa (1972) were the first to describe the purification of PHA by affinity chromatography. At the time they were using a sugar-starch matrix for lectin studies. This approach could not be used for kidney bean lectin because the only sugar residue bound by PHA is N-acetyl-D-glucosamine, and this only slightly. However, since PHA avidly

binds glycoproteins, porcine thyroglobulin coupled to sepharose was used. The PHA bound to this column was eluted with glycine-HCl buffer at pH 3.0. Felsted et al. (1975) also used the procedure introduced by Matsumoto and Osawa (1972) for PHA purification. This has proven to be an efficient method as the reported yield was 0.7 % compared to 0.2 % for ammonium sulphate fractionations (Jaffe, 1959).

Pusztai and Watt (1974) isolated pure PHA by chromatography on a fetuin-Sepharose 4B affinity support. Interestingly, these authors used an acetate equilibration buffer at pH 8.3 that included 0.001 M $CaCl_2$ and 5 x 10^{-5} M $MnCl_2$ ostensibly to aid the lectin-fetuin binding reaction. The material eluted from the column with 8 M urea was reported to be over 95 % pure PHA. Ohtani et al. (1980) also purified PHA with affinity chromatography, but they used a commercially available con A-Sepharose matrix. Elution of the purified PHA was accomplished with physiological saline containing 50 mM α-methyl-D-mannoside.

Hemagglutinating Activity. Since PHA is a potent agglutinator of erythrocytes, it is logical that a number of methods using red blood cells have been developed to analyze for it. The methods employed fall roughly into four categories: visual, spectrophotometric, electrical and biological. These will be discussed separately.

Methods based on visual assessment of agglutination are best described by the method of Jaffe and Gaede (1959). While investigating the hemagglutinin of black beans, they mixed 0.25 % mice erythrocytes in saline with the agglutinin. If after 30 minutes at 37°C followed by brief centrifugation at 2,500 r.p.m., the erythrocytes failed to resuspend on shaking, the agglutination test was considered positive. This test, although not quantitative, is good for rapidly estimating the

relative agglutinating strength of a protein.

A somewhat different visual method for determining the hemagglutinating ability of a substance is the type used by Felsted et al. (1975). The agglutinating activity of PHA was monitored in a microtiter plate by mixing one part of 4 % rabbit erythrocytes with 10 parts saline-diluted PHA. The agglutination test was positive if after 30 minutes at room temperature there was a carpet of red cells on the bottom of the microtiter well. This procedure has the same inherent drawback as the macro method of Jaffe and Gaede (1959) in that it is semi-quantitative at best.

Khole and Kauss (1980) also used a microtiter well technique almost identical to that used by Felsted and colleagues. The only difference between the two methods was Khole and Kauss's required five hours to reach equilibrium before visual assessment was performed.

Liener (1955) introduced a spectrophotometric method using trypsin treated rabbit erythrocytes. The red blood cells were diluted with an equal volume of hemagglutinin containing saline, allowed to stand for 2.5 hours and absorbance determined at 620 nm. The resulting data can then be correlated to a standard curve.

Kohle and Kauss (1980) were the first to describe the use of an electrical gating technique to count the red blood cells left in suspension after treatment with a hemagglutinin. Their procedure required the use of a laborious hanging drop technique and they used a Coulter Counter to determine the number of unagglutinated red cells as an index of agglutination. In this method, the erythrocytes are measured by their resistance to current flow through an aperture. This counting method is accurate and precise and the method of choice for

clinical blood testing.

Ohtani et al. (1980) described a quantitative PHA-glycoprotein precipitation for the determination of PHA. The PHA was incubated with Con-A, mucin or fetuin at $5^{o}C$ for three days and the precipitate that formed was dissolved in 0.5 N NaOH and the protein content determined by the Lowry-Folin method.

Although not working with PHA, Ghosh and co-workers (1979) developed a quantitative enzyme inhibition assay to determine the concentration of ricin, the lectin of R. communis. In this method, a ricin specific sugar (p-aminophenyl-galactopyranose) is conjugated to lysozyme near its active site. When ricin was added to the reaction mixture containing the modified lysozyme and its substrate, a reduction in enzyme activity occurred which was related to the ricin concentration. No similar work has been reported using PHA, but this probably could be accomplished. One necessary change would be the substitution of an inhibitory oligosaccharide for the sugar residue at the active site on lysozyme.

MATERIALS AND METHODS

Experimental Outline

The description of the methods employed in this study will be developed sequentially with a description of the overall thrust of the work, a more detailed look at the individual studies involved, an explanation of the treatment procedures and sample preparations and finally the detailed analytical methods used.

All of the work presented in this dissertation was designed to contribute to improving the biological quality and availability of dry beans (P. vulgaris). One major nutritional drawback to the consumption of dry beans is the presence of lectins. The studies comprising this dissertation were developed to answer questions about the stability of these proteins to various treatments and to indicate possible avenues of investigation that may lead to improved nutritional quality for bean consuming populations, especially in the developing countries.

Study One: Hemagglutination Assay

This study was undertaken to achieve three objectives: 1). to develop an improved hemagglutination assay using a cell counting instrument; 2). to determine the thermal stability of kidney bean lectin in whole beans; and 3). to determine the hemaglutinating activity of beans cooked at temperatures expected in either high altitude settings or crock-pot cookers.

The hemagglutination assay was developed using purified

phytohemagglutinin and saline extracts of raw kidney beans. The thermal stability of hemagglutinating activity of cooked kidney beans was determined using a cell counting method. In this experiment, beans were soaked for 12 hours and then heated in test tubes at 82^{o}, 88^{o}, 93^{o} and $100^{o}C$ for times ranging from 10 minutes to 8 hours. The lectins of the thermally processed beans were then extracted and the hemagglutination activity determined. To determine the stability of the hemagglutinating activity to low-temperature cooking procedures, whole dry beans were soaked overnight and cooked in a crock-pot cooker set at low and high settings. Samples were withdrawn periodically and evaluated for hemagglutinating activity and texture.

Study Two: PHA Molecular Study

The objective of this study was to determine the stability of purified PHA to a variety of thermal, chemical and enzymatic treatments. PHA from raw bean extracts was purified by affinity chromatography and the pure protein was subjected to thermal, chemical and enzymatic abuse. To study the effect of thermal treatment, PHA was diluted in saline and held for predetermined intervals at 70^{o}, 80^{o}, 90^{o} and $100^{o}C$. The heated samples were then analyzed for hemagglutinating activity and subjected to electrophoretic analyses. The effect of urea, mercaptoethanol, sodium chloride, acid and alkaline conditions on PHA stability was investigated by incubating the protein with these chemicals for 30, 60 and 120 minutes and then evaluating the hemagglutinating activity and finally subjecting it to electrophoretic analyses. Finally, the effect of enzymes on PHA was evaluated. Purified PHA (PHA-P) was incubated with a variety of proteolytic and carbohydrate digesting enzymes for one, two and three hours. Following

digestion, the hemagglutinating activity was determined and the reaction mixture analyzed by electrophoretic methods.

Study Three: Bean Processing Technology

The objective of this study was to evaluate the effect of non-traditional processing methods such as extrusion and alkaline cooking on the hemagglutinating activity and texture of dry beans. Whole beans and bean flours were extruded in a commercial extruder under variable conditions. Whole kidney beans were also subjected to soaking at neutral and high pH and cooked according to traditional means. The products from both these treatments were then assayed for hemagglutinating activity and analyzed by electrophoretic methods. In addition, the beans soaked and cooked at neutral and high pH were evaluated for textural changes.

Study Four: Breeding Line Study

This study was designed to evaluate 16 P. vulgaris varieties from the Michigan State University, small seed, dry bean nursery for a number of quality and protein characteristics. The beans were produced in experimental plots at Michigan State University during the 1984 growing season. The seeds were evaluated for protein, moisture, ash, color, hemagglutinating activity, digestibility and component peptides by the methods described further in this chapter.

Sample Preparation and Treatment

Source of Beans

The dry beans used in these studies came from a variety of sources. The kidney beans used in studies one and two were commercially

available dark red kidney beans (Montcalm variety, Michigan Foundation Seed, East Lansing , MI). The beans used in study three were the following: dark red kidney (Montcalm variety), black turtle soup type (Domino variety), pinto (Oletha variety). Finally, the P. vulgaris varieties used in study four were produced at The Michigan State University, Saginaw Valley Bean and Beet Research Farm during the 1984 growing season as part of the international, small seeded, dry bean, quality nursery. The seed pedigrees were: 800242, Sanilac, 8217-111-24, Nep-2, Black turtle soup, MSU-61380, 8217-VIII, Jalpataqua-72, San Fernando, Ica-Pijao, FF4-13-M-M-M-M, Jamapa, Protop-pi, Carioca, P766 and Mexico 12-1.

Thermal Inactivation (Study One)

Controlled heating studies were carried out according to the following schedule. Whole commercial dark red kidney beans (Michigan Foundation Seed, East Lansing, MI) were soaked overnight at $4^{o}C$ in physiological buffered saline (PBS) in an 18 x 175 mm test tube (beans:PBS, 1:10, W/V). The PBS used in all experiments contained 180 mEq Na^{+}/L, 5.1 mEq K^{+}/L, 153 mEq Cl^{-}/L, and 1 mM EDTA at pH 7.4. This solution was used instead of water to provide a standardized chemical composition, to provide a controlled osmolarity similar to cooking medium in home-cooked beans, and facilitate solubilization and extraction of PHA from the soaked and cooked beans. The tubes were placed in water baths at 82^{o}, 88^{o}, 93^{o} and $100^{o}C$ for times ranging from 10 minutes to 4 hours. The range of temperatures chosen included those expected in either a home-type crock-pot cooker or for high altitude open-kettle cooking. The zero time of this experiment was established when the samples were immersed in the constant temperature water bath.

The samples were removed from the bath at pre-determined intervals and immediately plunged into an ice-water bath for rapid cooling. The experiment was conducted in triplicate with duplicate determinations of hemagglutinating activity made on each sample.

Cooking (Study One)

Studies to simulate in-home and high altitude cooking were carried out in the following manner: Whole kidney beans (500 g) were added to PBS (beans:PBS, 1:5, W/V) and soaked overnight at $4^{o}C$. The soaked beans and excess PBS were transferred to crock-pot cookers with heating rates and final temperatures of (low setting) $13^{o}C/h:82^{o}C$ and (high setting) $14.5^{o}C/h:91^{o}C$. The zero of this experiment was established when the soaked beans and their cooking medium were introduced to the crock-pot cookers. The final temperature of the cookers at each setting was reached after six hours of steady heating. At hourly intervals, samples were withdrawn and rapidly cooled in an ice water bath to room temperature. This experiment was conducted in triplicate with duplicate determinations of hemagglutinating activity made on each sample.

Affinity Chromatography Purification (Study Two)

Phytohemagglutinin (PHA) was purified by concanavalin A-agarose affinity chromatography. For purification, bean extracts were added to the concanavalin A-agarose gel and allowed to stand for one hour. The non-bound protein was washed off the gel with distilled water and saline at pH 7.2 until absorbance at 254 nm had stabilized. Removal of the bound PHA was accomplished by elution with PBS containing 0.1 M Methyl-d-mannoside. The sugar was removed from the gel by elution with pH 3.5 Acetate buffer containing 2 M sodium chloride. The PBS used contained 180 mEq Na^{+}/L, 5.1 mEq K^{+}/L, 153 mEq Cl^{-}/L with 1 mM EDTA at pH 7.2.

The purified PHA protein (PHA-P) was then pervaporated, freeze dried and stored frozen for later use.

Thermal Treatment (Study Two)

The PHA was diluted with PBS (5.0 mg/mL) and subjected to the following thermal treatments: 70^{o}, 75^{o} and 80^{o}C for two, four and eight hours; 85^{o}C for one, two and four hours; 90^{o}C for 15, 30, 45 and 60 minutes; and 100^{o}C for 5, 10, 15, 20, 25 and 30 minutes. Triplicate samples were run and duplicate evaluations were made on each.

Chemical Treatment. (Study Two)

Purified PHA was diluted with PBS (5.0 mg/mL) and subjected to the following chemical treatments: 2 M NaCl, 5M urea, 5% mercaptoethanol, pH 12.0 and pH 3.0. Triplicate samples were run and duplicate determinatinations made on each sample.

Enzymatic Treatment. (Study Two)

The purified PHA was diluted with PBS (5.0 mg/mL) and subjected to digestion with the following enzymes (all from Sigma Chemical Co., St. Louis, MO): Pepsin (EC 3.4.23.1, from porcine stomach mucosa), Trypsin (EC 3.4.21.4, from porcine pancreas), Chymotrypsin (EC 3.4.21.1, from bovine pancreas), Peptidase (from porcine intestinal mucosa), Protease (from Streptomyces griseus), Pancreatin (from porcine pancreas, grade VI), Alanine Amino Peptidase (from bovine intestinal mucosa), Neuraminidase (from Clostridium perfringens, type VIII), α-amylase (1,4-a-D-Glucan glucanhydrolase; EC 3.2.1.1), β-amylase (1,4-a-Glucan maltohydrolase; EC 3.2.1.2) and α-Mannosidase (from Jack Beans). In each case the PHA was digested for one, two and three hours and the digestion mixture assayed for hemagglutinating activity and analyzed by electrophoresis. Triplicate samples were run and duplicate

determinations made on each sample.

Extrusion (Study Three)

Commercially available kidney (Montcalm variety) and black beans (Domino variety) were ground in a Udy grinder to a 100 mesh flour. The flours and whole beans were passed through a Creusot-Loire Model 2000 extruder (Usine de L'Ondaine Firminy, France) under varying temperature and water feed conditions. Extruded products were cut at lengths of 6 to 10 mm by an air driven blade, dried for 24 hours at 38°C and stored in polyethelyne bags at 4°C.

Alkaline Cooking (Study Three)

Whole kidney beans were soaked in PBS adjusted to pH 12 with NaOH and cooked at 77° and 93°C for 2, 4 and 8 hours. Beans were immediately cooled to 0°C following cooking, extracted with PBS and the extract assayed for hemagglutinatinating activity and evaluated by electrophoresis. This experiment was done in triplicate with duplicate determinations.

Analytical Methods

Protein

Micro-Kjeldahl. Approximately 30 mg of bean flour were weighed and analyzed by the standard micro-kjeldahl procedure. Percent Nitrogen obtained was then multiplied by 6.25 to obtain % protein (AACC Method 46-13):

$$\% \text{ Total Protein} = \% \text{ Nitrogen} \times 6.25$$

Protein Solubility Index. Approximately 5 g of bean flour were mixed with 40 mL of PBS at 4°C overnight. The mixture was then centrifuged to obtain a clear supernatant. The percent nitrogen of the clear supernatant was then determined using AACC Method 46-13. The protein solubility index , expressing soluble nitrogen as a percentage of of the total nitrogen, was calculated as follows:

(% Soluble Nitrogen/% Total Nitrogen) x 100

Lowry. The Lowry protein determination is a two step reaction, in which soluble protein forms a complex with copper in an alkaline medium that rapidly reduces to yield an intense blue color. Protein concentration was determined by comparison with a standard curve, produced using a commercially available lectin (Phytohemagglutinin, Sigma Chemical Co., St. Louis, MO).

Moisture

Oven Drying. Approximately 5 g of bean flour were weighed onto previously dried and tared crucibles and dried to a constant weight at 80°C for 24 hours in an air oven. Percent moisture was determined by weight loss on a fresh weight basis (AACC Method 44-15).

% Moisture = (Moisture Loss (g)/Sample Fresh Weight (g)) x 100

Dielectric Moisture Meter. The dielectric moisture meter determination is applicable to all grains for which conversion charts are available. A 200 g sample of seed was weighed, the temperature of the sample recorded and the moisture determined using the Motomco Moisture Meter Model 919 (Motomco, Inc., Clark, NJ). Percent moisture of the seeds was then calculated from prepared charts. (AACC Method 44-11).

Ash

Dried samples obtained from the above moisture determination were placed in a muffle furnace at 525°C for 24 hours. The uniform white ash was cooled in a dessicator and weighed at room temperature. Percent ash was determined on a dry weight basis (AACC Method 08-01).

Percent Ash = (Residue Weight (g)/Sample Dry Weight (g)) x 100

Hunterlab Color Values

The Hunterlab Model D25-2 Color/Difference Meter (Hunter Associates Laboratory, Inc., Reston, VA) standardized with a white tile (L = 95.35, a_L = -0.6, b_L = +0.4) was used to evaluate the color of dry bean varieties. A 100 g sample of dry beans was placed in an optically inert glass cup and covered with an inverted white lined can to shield the sample port from extraneous light.

Lectin Purification

Ammonium Sulphate Precipitation. Concentrated lectin fractions were precipitated from bean extracts by the method of Jaffe and Hannig (1965). One kg of bean flour was extracted overnight at 4°C with 5 L of physiological buffered saline. Ammonium sulphate was added to the bean extracts to a level of 75 % saturation. The precipitate produced was collected by centrifugation, resuspended in distilled water and precipitated again with 75 % Ammonium Sulphate. The precipitate collected was dialyzed against several changes of distilled water at 4°C, pervaporated and freeze dried or stored frozen for later use.

Electrophoretic Separations

Discontinuous Polyacrylamide Gel Electrophoresis (DISC-PAGE). DISC-PAGE was used to resolve the component peptide patterns of the treated PHA-P. The method used was that of Davis (1964) but staining

was accomplished with 0.04% Coomassie Brilliant Blue G-250 in 3.5 % perchloric acid overnight. For all DISC-PAGE evaluations, 11 % acrylamide gel concentration was used for the running gel and the proteins were subjected to 1 mA/tube for 10 minutes followed by 3 mA/tube for the remainder of the separation. The gels were destained and stored in 7 % aqeous acetic acid.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was performed to resolve the component peptides of the treated samples according to their molecular weight. The method used was that of Weber and Osborne (1969). For all SDS-PAGE evaluations, 10 % acrylamide gel concentration was used and the gel tubes were allowed to polymerize for 24 hours before use. The tubes were subjected to 3 mA/tube for 10 minutes followed by 8 mA/tube for the remainder of the separation. After development, the gels were stored in 7 % acetic acid. A series of molecular weight standards were run along with the bean extracts and treated PHA-P. The protein standards were obtained from Sigma Chemical Co. (St. Louis, MO) and their molecular weights in daltons were: Phosphorylase B (94,000); Bovin serum albumin (67,000); Ovalbumin (43,000); Carbonic anhydrase (30,000); Soybean trypsin inhibitor (20,000); and α-Lactalbumin (14,400).

Hemagglutinating Activity

Preparation of Bean Extracts. The hemagglutinating activity was determined by soaking raw whole kidney beans overnight (beans:PBS, 1:10, W/V) at 4^{o}C. After soaking, the beans were ground in a Waring Blendor, and centrifuged (40,000 x g, 30 minutes). The saline supernatant was decanted and assayed for hemagglutinating activity either on the same day or frozen and assayed on the following day. Extracts of cooked

beans were prepared on a constant solids basis as raw extracts.

Preparation of the Red Blood Cell Suspension. Porcine whole blood was collected and washed three times with PBS to remove soluble blood constitutents. The sensitivity of the erythrocytes to agglutination was increased by incubation with 0.005% trypsin (lyophilized powder from porcine pancreas, type IX, Sigma Chemical Co., St. Louis, MO) for one hour at 35°C. Following incubation, the treated cells were washed three times with PBS and either suspended in PBS for the agglutination assay or packed by centrifugation (400 x g, 45 seconds) and stored at 4°C for later use. Treated erythrocytes were diluted with PBS to give a red blood cell (RBC) suspension having a machine cell count of 4×10^7 (4×10^8 cells/mL) using a Coulter Counter Model ZB-I (Coulter Electronics, Inc., Hialeah, FL). Standardized instrument settings were: 1/Amp = 1/2; 1/aperature current = 1; matching switch = 20,000; gain = 6.5; manometer volume = 0.1 mL; lower threshold = 10; upper threshold = off. Treated and packed cells were stable at 4°C for up to five days. Fresh RBC suspension was prepared daily for all experimentation.

Hemagglutination Assay. Purified lyophilized PHA (Phytohemagglutinin M, P.L. Biochemicals, Milwaukee, WI) was made to a concentration of 10,000 ppm in PBS daily as a standard. Two mL aliquots of RBC suspension were mixed with freshly prepared PHA standard or bean extract in a 75 x 10 mm test tube and allowed to stand for one hour. The mixtures were then packed by cenrifugation (400 x g, 45 seconds), resuspended by shaking, and allowed to stand for 15 minutes. At this time, 10 uL were drawn from the midpoint of the sample tubes and added to 10 mL PBS. The diluted sample was then assayed using a Coulter Counter to determine the number of erythrocytes remaining in suspension.

In-Vitro Digestibility

Sample Preparation. Samples used included a lot of 18 dry bean varieties which were assayed for protein digestibility raw and after cooking. Raw beans were soaked in distilled water (water/bean, 3:1, v/w) for 18 hours at 5°C. Soaked beans were cooked in the autoclave (15 psi, 121° C) for 30 minutes. Cooked beans were dried in a forced draft oven at 60°C for 18 hours, ground in a Udy cyclone mill and analyzed for protein content by the micro-kjeldahl method.

Procedure. The in-vitro digestibility of the samples was assessed by measuring the extent to which the pH of the protein suspension dropped when treated with a multienzyme system (all enzymes obtained from Sigma Chemical Co., St. Louis, MO) including trypsin (porcine pancreatic, Type IX), chymotrypsin (bovine pancreatic, Type II), peptidase (porcine intestinal) and Streptomyces griseus protease (Type XIV), to complete proteolysis (Wolzak, 1981a). An amount of sample containing 250 mg of crude protein was weighed into a 100 mL beaker and 40 mL of distilled water were added. The protein suspensions were placed in the refrigerator for two hours before analysis. The enzyme solutions were freshly prepared before each series of tests. The multienzyme solution contained 22,704 BAEE units of trypsin, 186 units of chymotyrypsin and 0.052 units of peptidase per mL. This solution was adjusted to pH 8.00 with 0.1 N NaOH and/or HCl while stirring in a 37°C water bath. The protease solution contained 7.95 mg/mL. Both were distributed into tubes containing the amount needed for each assay (4 mL) and placed in an ice bath until used.

Each sample was placed in a 37°C water bath and adjusted to pH 8.00

with 0.1 N NaOH and/or HCl, while stirring constantly with a magnetic stirrer. The multienzyme solution was then added. The pH drop was measured with an Orion Research microprocessor ionalyzer/901 (Orion Research, Cambridge, MA). At exactly 10 minutes of digestion, the bacterial protease solution was added and water of a 55°C water bath circulated to the sample. The pH at 15 minutes of total digestion was recorded and the in-vitro digestibility calculated according to the formula:

In-vitro digestibility = 289.677 - 32.811 (pH 15 min)

proposed by Wolzak et al. (1981a and b) for P. vulgaris. Sodium caseinate was used as a reference sample with each series of tests. Duplicate runs of each sample were done.

Texture

Kramer Shear Press. (Model TR3 Texturecorder, Food Technology Corp., Rockville, MD). The texture of the cooked beans was determined objectively by shearing a 100 g portion of the cooked sample in an Allo-Kramer shear press equipped with a 3000 pound transducer and a number C-15 standard multi-blade shear compression cell. Shear peak heights indicating maximum force to shear cooked beans were recorded and expressed as Kg/g bean.

Subjective Textural Evaluation. The texture was also subjectively evaluated for tenderness by a trained panel of six judges. The judges were knowledgable about the textural characteristics of cooked and canned dry beans and evaluated the cooked product in an open laboratory. During duplicate cooking cycles, judges were presented 50 g samples of beans withdrawn from the cookers at specified time intervals. Using a single sample presentation, panelists judged the

beans to be palatable or not palatable based on masticatory tenderness. The point of palatability was defined as the time required for 50 % of the responses to indicate that the beans were tender enough to be eaten.

Statistical analysis

The "Statistical Package for the Social Sciences" (SPSS) computer programs were used on a CDC 6500 computer to assist the statistical analysis. Analysis of variance was used to determine the effect of treatments using the SPSS subprogram ANOVA. Treatment means were analyzed for significant differences at the $P < 0.05$ and $P < 0.01$. Mean squares with significant F ratios were reported with probability levels of $P < 0.05$ (*) and $P < 0.01$ (**).

STUDY ONE: THERMAL INACTIVATION OF THE HEMAGGLUTINATING ACTIVITY OF LOW TEMPERATURE COOKED KIDNEY BEANS

Introduction

Dry beans are an important food crop which could supply populations of less developed countries with a major portion of their protein and nutrients. However, the presence of antinutrients is a disadvantage to legume consumption. Of a number of antinutrients in dry beans, one of the most toxic is phytohemagglutinin (PHA), the lectin of kidney beans (Jaffe and Vega Lette, 1968; Honavar et al., 1962; Putsztai and Palmer, 1977; Pusztai et al., 1981; King et al., 1980a, 1980b; Wilson et al., 1980). Phytohemagglutinin is a tetrametric glycoprotein that exists as a mixture of five hybrids with similar chemical properties, but slightly different biological activities (Leavitt et al., 1977). PHA has potent biological activity because of its ability to bind complex carbohydrates and other glycoproteins. Some of the adverse effects of PHA are: 1). the agglutination of erythrocytes and bacteria; 2). binding to intestinal epithelium; and 3). the stimulation of lymphocyte formation.

The toxicity of the kidney bean and its lectin, PHA, has been recognized for some time. Consumption of uncooked kidney beans resulted in depression of growth, weight loss, and death in rats (Jaffe and Vega Lette, 1968; Honavar et al., 1962; Rattray et al., 1974; Pusztai and Palmer, 1977; Pusztai et al., 1981) and birds (Hewitt et al., 1973;

Jayne-Williams and Burgess, 1974). Although the toxicity of PHA is well documented, the mechanism for toxicity is not known. PHA releases amylase and lipase from binding sites on the glycocalyx of the intestinal epithelium (Sandholm and Scott, 1979). It also inhibits activity of saccharase, (Rouanet and Besancon, 1979) and brush border dipeptidases (Kim et al., 1976). This interference with intestinal enzyme activity may result in decreased nutrient digestion and absorption. It has also been shown that feeding pure PHA to rats binds to and disrupts the intestinal epithelium leading to the formation of abnormal microvilli and damaged epithelial surfaces (King et al., 1980a, 1980b). The PHA-induced epithelial damage promotes an overgrowth of non-hemolytic E. coli in the small intestine which may be responsible for the toxicity induced by consumption of raw kidney beans (Wilson et al., 1980).

Toxicity of raw kidney beans in man has also been reported. In a review of food poisonings in Britain, seven outbreaks were attributed to kidney beans (Noah et al., 1980). In every case, the onset of symptoms of nausea, vomiting, diarrhea, and abdominal pain was rapid (one to three hours). Two of these outbreaks were caused by cooked beans. In one instance, the beans were casseroled in an oven set at 150-160^{o}C for three hours and in another, cooked at a low setting in a crock-pot cooker for 5.5 hours.

Previous reports have utilized a variety of cell-agglutination techniques for measuring lectin activity. These include microscopic or macroscopic visual observation of cells in microtiter wells (Felsted et al., 1975) and spectrophotometric quantitation of suspended erythrocytes (Liener, 1955). These methods have been extensively employed for

determining lectin activity. Although these are sensitive and rapid, the proposed method of sample preparation and quantitation enables the determination of low lectin activity using a commonly available cell counting instrument. A number of workers (Davis, 1981; Kohle and Kauss, 1980; Oppenheimer and Odencrantz, 1972) have reported the reliability and sensitivity of cell agglutination assays using electronic cell counting techniques. According to Davis (1981), a particular advantage of using a cell counter to determine lectin activity is it requires only ug quantities of these active proteins.

This paper reports a sensitive cell-counting instrumental technique for measuring the hemagglutinating activity of whole kidney beans prepared under low-temperature cooking conditions.

Materials and Methods

Thermal Inactivation

Controlled heating studies were carried out according to the following schedule. Whole commercial dark red kidney beans (Michigan Foundation Seed, East Lansing, MI) were soaked overnight at $4^{o}C$ in physiological buffered saline (PBS) in an 18 x 175 mm test tube (beans:PBS, 1:10, W/V). The PBS used in all experiments contained 180 mEq Na^{+}/L, 5.1 mEq K^{+}/l, 153 mEq Cl^{-}/L, and 1 mM EDTA at pH 7.4. This solution was used instead of water to provide a standardized chemical composition, to provide a controlled osmolarity similar to cooking medium in home-cooked beans, and to facilitate solubilization and extraction of PHA from the soaked and cooked beans. The tubes were then placed in water baths at 82^{o}, 88^{o}, 93^{o} and $100^{o}C$ for times ranging from

10 minutes to four hours. The range of temperatures chosen included those expected in either a home-type crock pot cooker or for high altitude open kettle cooking. The zero time of this experiment was established when the samples were immersed in the constant temperature water baths. The samples were removed from the bath at pre-determined intervals and immediately plunged into an ice-water bath for rapid cooling. The experiment was conducted in triplicate with duplicate determinations of hemagglutinating activity made on each sample.

Cooking

Studies to simulate in-home and high altitude cooking were carried out in the following manner: whole kidney beans (500g) were added to PBS (beans:PBS, 1:5, W/V) and soaked overnight at $4^{o}C$. The soaked beans and the excess PBS were transferred to crock-pot cookers with heating rates and final temperatures of (Low setting) $13^{o}C/h$:$82^{o}C$ and (High setting) $14.5^{o}C$:$91^{o}C$. The zero time of this experiment was established when the soaked beans and their cooking medium were introduced to the crock pot cookers. The final temperature of the cookers at each setting was reached after six hours of steady heating. At hourly intervals, samples were withdrawn and rapidly cooled in an ice water bath to room temperature. This experiment was conducted in triplicate with duplicate determinations of hemagglutinating activity made on each sample.

The texture of the cooked beans was determined objectively by shearing a 100g portion of the cooked sample in a Kramer shear press equipped with a multi-blade shear compression cell. Shear peak heights, indicating maximum force to shear cooked beans, were recorded and expressed as kg/g bean. The texture was also subjectively evaluated for tenderness by a trained panel of six judges. The judges were

knowledgable about the textural characterisitics of cooked and canned dry beans and evaluated the cooked product in an open laboratory. During duplicate cooking cycles, judges were presented 50g samples of beans withdrawn from the cookers at specified time intervals. Using a single sample presentation, panelists judged the beans to be palatable or not palatable based on masticatory tenderness. The point of palatability was defined as the time required for 50 % of the responses to indicate that the beans were tender enough to be eaten.

Preparation of Bean Extracts

The hemagglutinating activity was determined by soaking raw whole kidney beans overnight (beans:PBS, 1:10, W/V) at 4°C. After soaking, the beans were subjected to various heat treatments, ground in a Waring Blendor, and centrifuged (40,000 x g, 30 minutes). The saline supernatant was decanted and assayed for hemagglutinating activity immediately or frozen and assayed on the following day. Extracts of cooked beans were prepared on a constant solids basis as raw extracts.

Preparation of the Red Blood Cell Suspension

Porcine whole blood was collected and washed once with PBS to remove soluble blood constituents. The sensitivity of the erythrocytes to agglutination was increased by treatment with trypsin (lyophilized powder from porcine pancreas, type IX, Sigma Chemical Co., St. Louis, MO.). Following incubation, the treated cells were washed three times with PBS and either suspended in PBS for the agglutination assay or packed by centrifugation (400 x g, 45 seconds) and stored at 4°C for later use. Treated erythrocytes were diluted with PBS to give a red blood cell (RBC) suspension having a machine cell count of 4×10^7 (4×10^8 cells/mL) using a Coulter Counter Model ZB-I (Coulter Electronics

Inc., Hialeah, FL). Standardized instrument settings were 1/Amp = 1/2; 1/aperture current = 1; matching switch = 20,000; gain = 6.5; manometer volume = 0.1 mL; lower threshold = 10; upper threshold = off. Treated and packed cells were stable at $4^{o}C$ for up to five days. Fresh RBC suspension was prepared daily for all experimentation.

Hemagglutination Assay

Purified lyophilized PHA (Phytohemagglutinin M, P.L. Biochemicals, Milwaukee, WI) was made to a concentration of 10,000 ppm in PBS daily as a standard. Two mL of RBC suspension were mixed with aliquots of freshly prepared PHA standard or bean extract in a 75 x 10 mm test tube and allowed to stand for one hour. The mixtures were then packed by centrifugation (400 x g, 45 seconds), resuspended by shaking, and allowed to stand for 15 minutes. At this time, 10 uL were drawn from the midpoint of the sample tubes and added to 10 mL PBS. The diluted sample was then assayed using a Coulter Counter to determine the number of erythrocytes remaining in suspension.

Results and Discussion

A logarithmic reduction in unagglutinated cells with increasing PHA levels between 0.05 - 0.15 mg PHA per mL RBC suspension is illustrated in Figure 4. The data showed little agglutination at PHA levels less than 0.05 mg/mL. At levels greater than 0.15 mg PHA mL, further increases in PHA did not result in further increase in agglutination. This asymptotic relationship at approximately 1000 cells/mL existed for every case using this assay method with various lectin sources.

At very low levels (<0.05 mg PHA/mL RBC suspension), the PHA

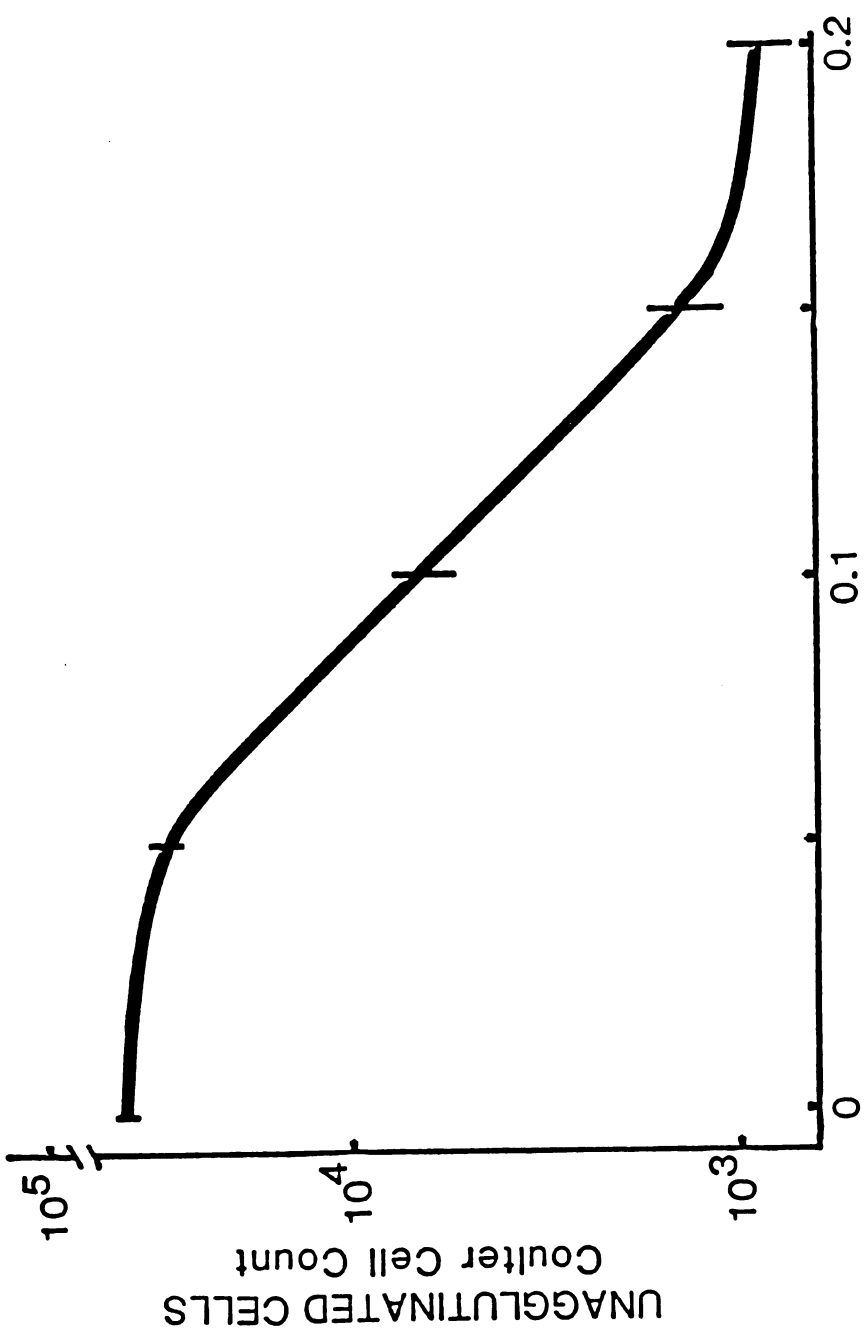


Figure 4. Agglutination of porcine erythrocytes by commercial Phytohemagglutinin (Y = -296412X + 42746 over range of 0.05 - 0.15 mg PHA/mL RBC suspension)

concentration was apparently inadequate to stimulate the agglutination and to precipitate erythrocytes. The decrease in reaction velocity at cell counts of approximately 1000 or less (PHA levels greater than 0.15 mg/mL) was indicative of mass action whereby erythrocytes left in suspension were too dilute and failed to collide with sufficient frequency to agglutinate and precipitate completely from the reaction mixture. Further, low counts may be attributable to traces of nonagglutinating blood components such as lymphocytes that were not removed from the whole blood during the washing procedure.

The sensitivity of this method was shown to enumerate lectin activity in the range 0.05 - 0.15 mg with a coefficient of variation (CV % = s/Y x 100) of less than 6 % over this range. This demostrates reliable data with greatest sensitivity of the method at approximately 0.1 mg activity. This methodology using porcine erythrocytes was also demostrated to be useful for human and rat but not bovine erythrocytes (these data not shown).

Thermal Inactivation

PHA activity decreased with increased heating time and/or heating temperature (Figure 5). These data illustrate increasing levels of cooked bean extract required to yield an agglutination response (decrease in coulter cell count). Hemagglutinating activity was not demostrated at the time/temperature combinations yielding no appreciable decrease in initial Coulter cell count. The following combinations were representative of zero hemagglutinating activity: 4 hours at 88^{o}C; 2 hours at 93^{o}C; and 30 minutes at 100^{o}C. Beans held at 82^{o}C showed less reduction in their hemagglutinating activities than those held at the higher temperatures. For beans held for one hour, the concentration of

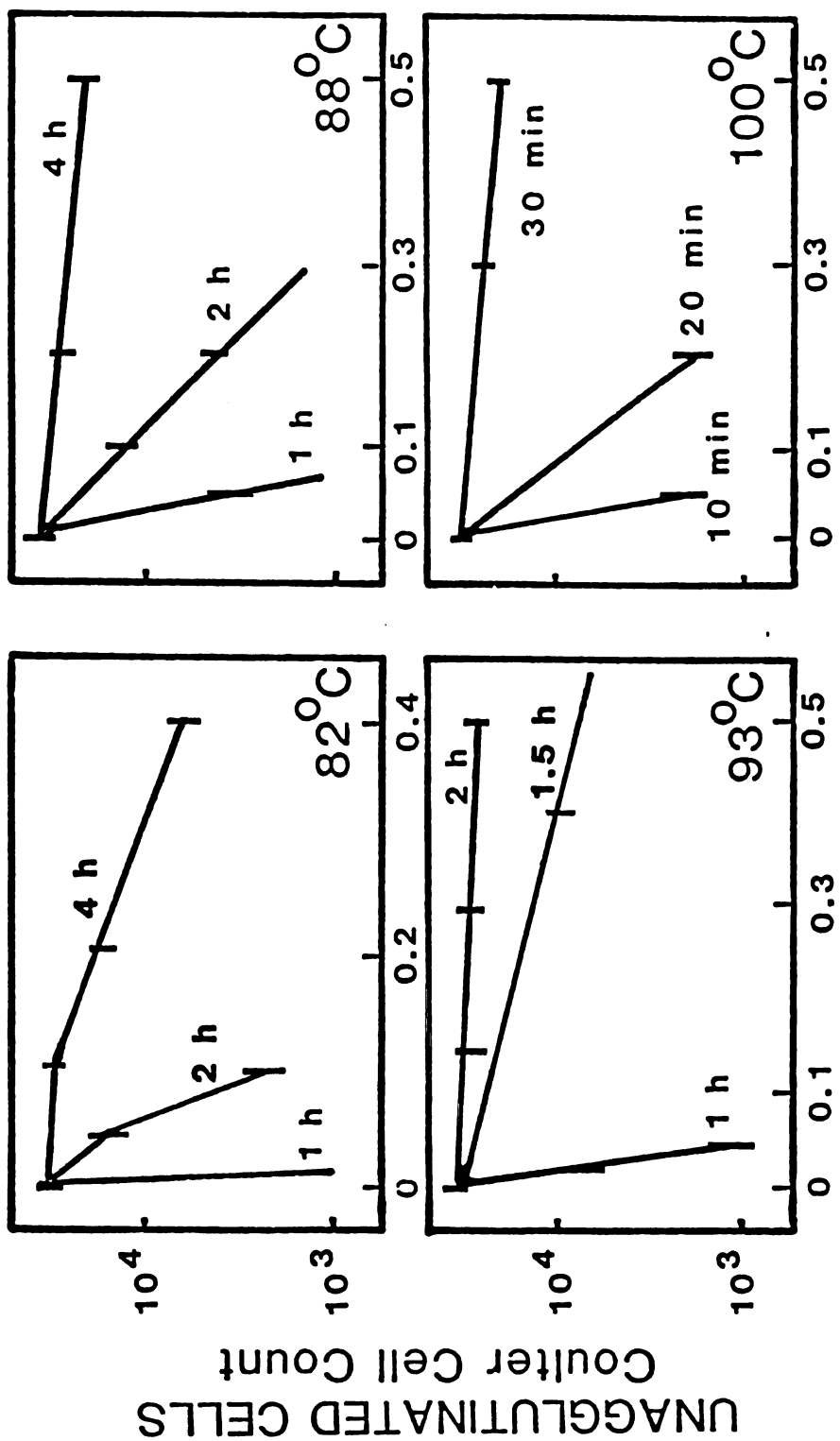


Figure 5. Agglutination of porcine erythrocytes by extracts of cooked kidney beans

an extract required to reduce the original RBC count by 50 % was 0.003 mL extract/mL RBC suspension. For those held two hours, the concentration of bean extract needed to produce the equivalent decrease in RBC count was 0.054 mL extract/mL RBC suspension; and for beans held for four hours, the amount required was 0.223 mL extract/mL RBC suspension.

Beans held at 100°C for 10 or 20 minutes retained some of their ability to agglutinate RBC's. However, 30 minutes at this temperature was sufficient to inactivate almost all dectectable PHA activity. Individual bean variation, such as size, density and hydration, had a greater effect on heat penetration during short time/high temperature cooking than during long time/low temperature cooking.

The thermal inactivation of the active protein is illustrated in Figure 6, by plotting the slopes of each line against time within temperature treatments (Figure 5). The time required to inactivate the glycoprotein responsible for RBC agglutination can be estimated from this plot. Thus, 160, 136, 62, and 12 minutes were required to reduce the activity of PHA by one log cycle at 82°, 88°, 93° and 100°C, respectively.

These time requirements for log reduction in PHA activity, when plotted against temperature, indicate the relative thermal inactivation of hemagglutinatig activity in whole kidney beans (Figure 7). For each 5.6°C increase in temperature, a reduction of 50 minutes holding time achieved the same level of PHA inactivation. Conversely, for each 5.6°C decrease in temperature, an additional 50 minutes were required to achieve the same reduction in PHA activity.

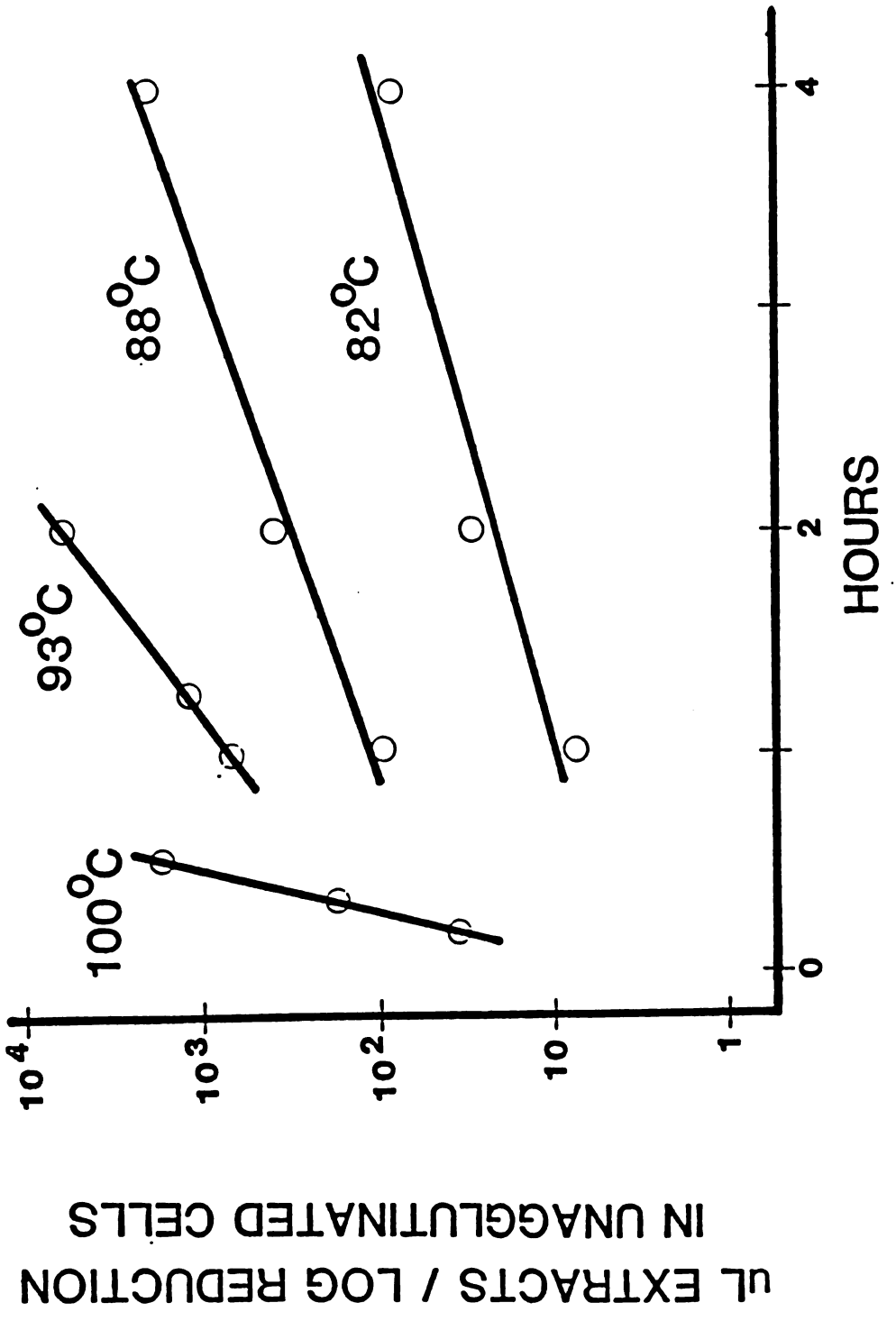


Figure 6. Relative efficiency of thermal treatments for PHA inactivation
82: Y = 0.157X - 0.193 88: Y = 0.375X - 0.404
93: Y = 3.904X - 4.460 100: Y = 5.530X - 1.160

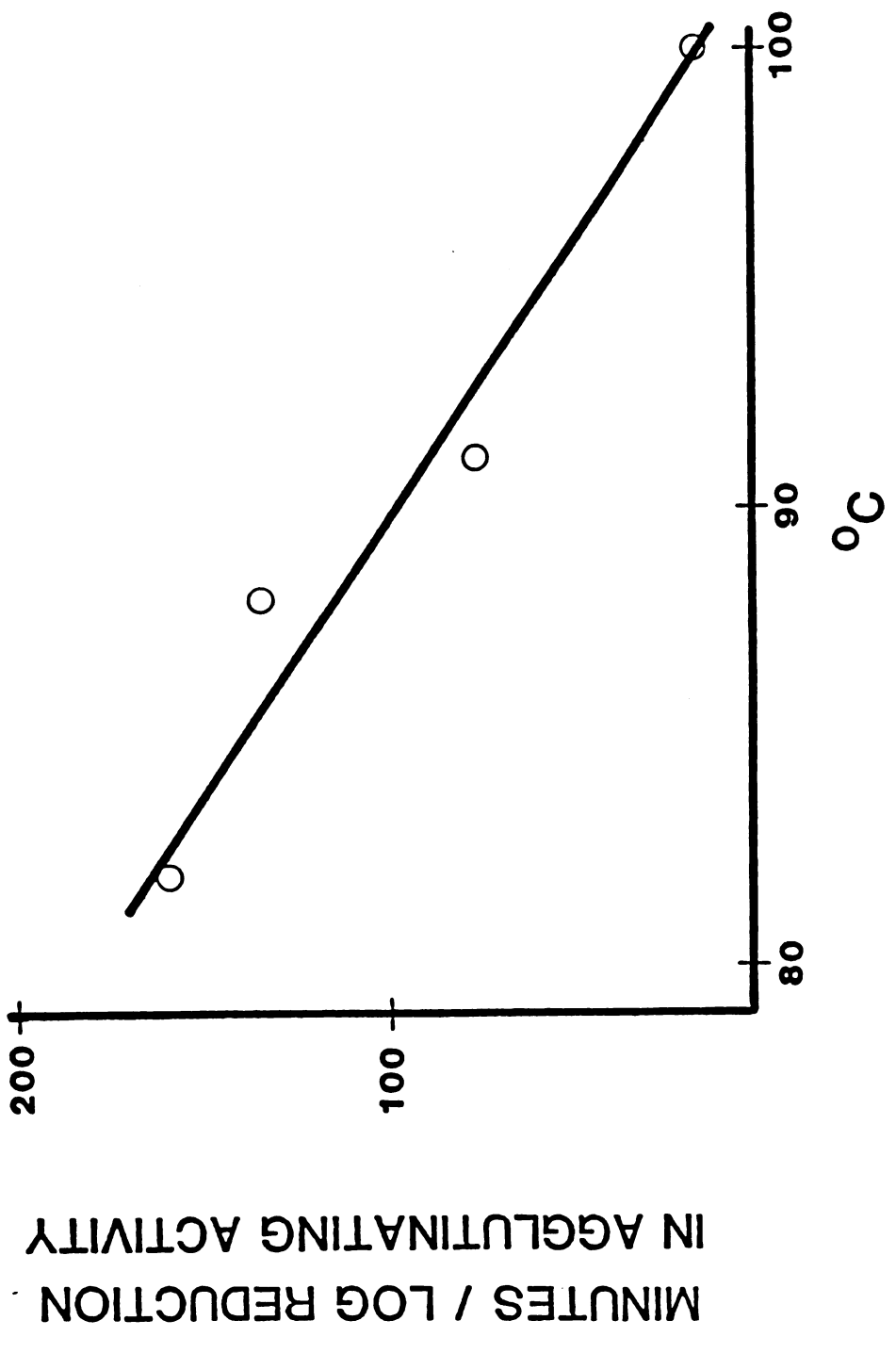


Figure 7. Thermal inactivation of hemagglutinating activity Y = -0.329X - 27.400

Low-temperature Cooking Study

Monitoring the hemagglutinating activity of beans cooked at low temperature is important for two reasons. First, there is evidence indicating that uncooked beans soaked at a low temperature (29°C) were toxic, even if marginally palatable (Public Health Laboratory Service, 1976). Also, kidney beans caused an outbreak of food poisoning even after cooking for 5.5 hours in a crock-pot (Noah et al., 1980). Although the crock-pot cooking temperature was not given, it probably did not exceed 82°C and may have been much lower. Second, beans are also a staple food in many lesser developed regions of the world such as Mexico, Central and South America. In those regions, much higher than sea level, bean cooking water boils at temperatures well below 100°C. For examples, the boiling point of water in Mexico City is 89°C. Thus, the experimental conditions chosen simulated normal crock-pot preparation and high-elevation open-kettle cooking temperatures.

The hemagglutinating activity of beans decreased with cooking time (Figure 8). In spite of this, detectable levels of hemagglutinating activity remained even after 11 hours of heating at 82°C. However, a 14 hour cooking time rendered the beans essentially free of PHA activity. Hemagglutinating activity was detected in beans held for five hours or less at 91°C. However, when the sample was held for six hours or more, no hemagglutinating activity was detected. The prescence of detectable levels of hemagglutinating activity following long holding times at low cooking temperatures indicated that the possibility existed for the chronic consumption of low levels of PHA in the diets of consuming populations. It should be noted that these temperatures represent final end point temperatures within the cooking bean mass at each setting.

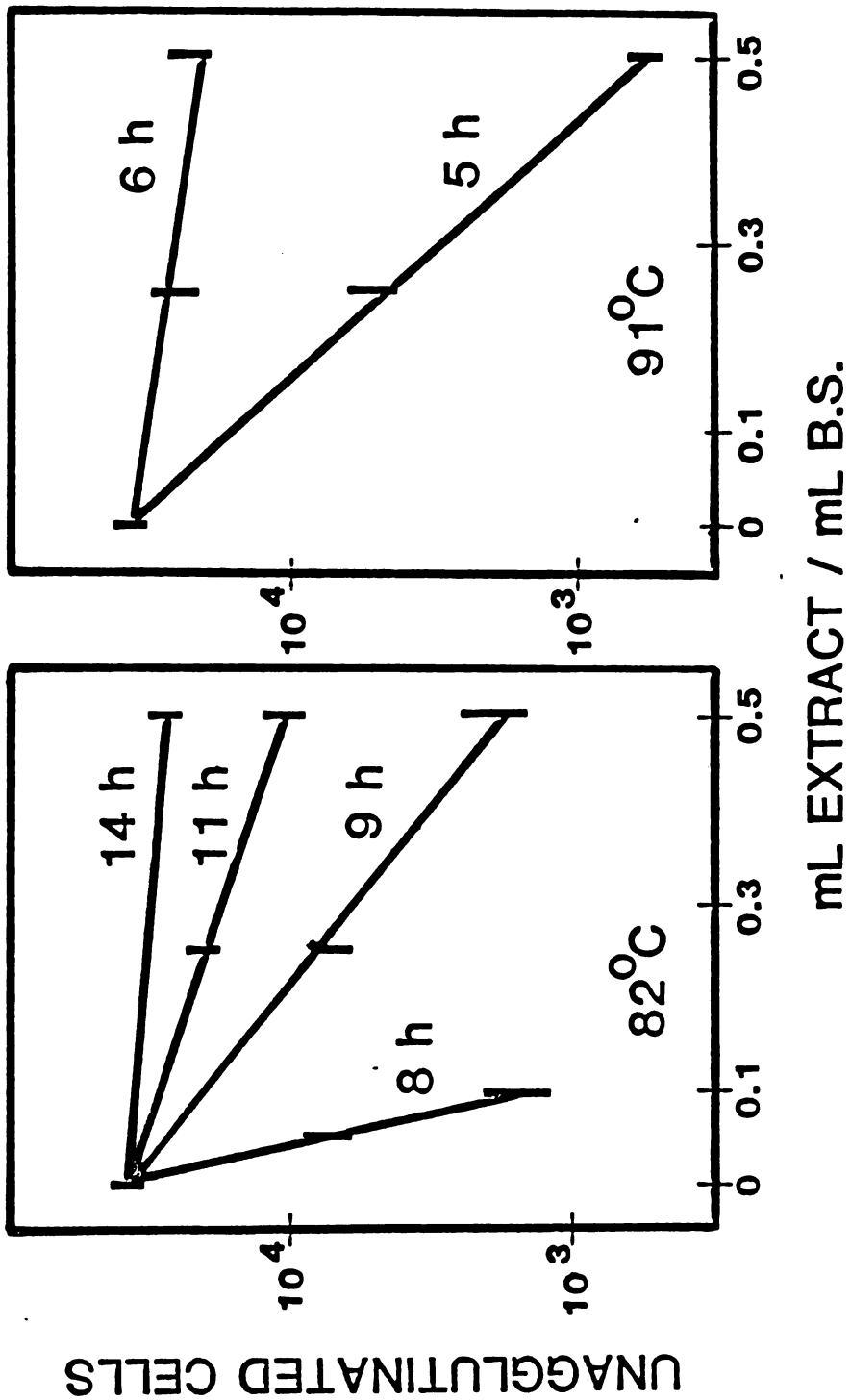


Figure 8. Agglutination of porcine erythrocytes by extracts of kidney beans cooked in crock-pots. Cooking was conducted at low (82°C) and high (91°C) settings with 5 x volume of PBS to bean weight

The heating rates for these two conditions were 13°C/h and 14.5°C/h for low and high settings, respectively and both nominal end point temperatures were reached after six hours of continuous heating. These conditions do not represent continuous heating at these temperatures but rather represent the normal heating pattern expected for casserole-style bean preparation.

Bean texture plotted against cooking time permits the simultaneous evaluation of palatability and hemagglutinating activity. Beans evaluated during cooking by a trained panel provided the point of 50 % acceptability for consumption. This point was designated the threshold limit for acceptability for consumption i.e., beans tender enough to be eaten. This threshold limit corresponded to a Kramer Shear Press value of 1.13 kg/g cooked bean. Bean samples requiring a force in excess of 1.13 kg/g to shear were considered too firm to be palatable. Shear resistance of beans cooked at 91°C intersected the minimum palatability line at 6 hours which was also sufficient to completely inactivate the hemagglutinating activity (Figure 9). However, the shear resistance of the beans cooked at 82°C intersected the palatability line at approximately 11.5 hours but hemagglutinating activity was detectable for up to 14 hours.

Thompson et al. (1983) employed controlled heating experiments utilizing relatively small samples to allow rapid establishment of designated temperatures similar to the conditions used in our thermal inactivation study. Based on the temperature range and the heating rates employed, our results are in general agreement with those of previous authors. However, the conditions utilized in the low-temperature cooking study incorporate a large bean mass and slow heating

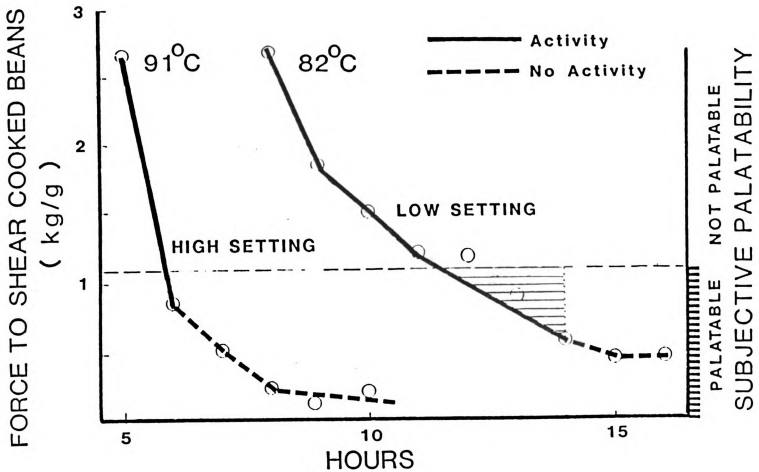


Figure 9. Relationships among bean texture, cooking condition and hemagglutinating activity for low temperature cooked kidney beans

rates to duplicate actual methods of home bean preparation. These data indicating presence of hemagglutinating activity in palatable cooked beans have not been demostrated before, although they support the documented toxicity of kidney beans cooked in crock-pots for long periods (Noah et al., 1980).

Conclusions

The electronic cell counter was a sensitive tool for measuring the hemagglutinating activity of kidney beans. The PHA in cooked kidney beans is usually thought to be inactivated by cooking. However, hemagglutinating activity was found in beans exposed to low temperatures even though the cooking times were greater than 12 hours. These data by design do not indicate the nutritional quality of beans cooked to eating softness at low temperatures but may have important nutritional implications. Further research is needed to evaluate any nutritional or physiological impairment resulting from chronic consumption of bean diets containing low levels of active PHA.

STUDY TWO: STABILITY OF PURIFIED PHYTOHEMAGGLUTININ TO THERMAL, CHEMICAL AND ENZYMATIC ABUSE

Introduction

Phytohemagglutinin (PHA), the lectin of kidney beans, is a toxic carbohydrate-binding glycoprotein. The toxicity of PHA to man and animals is well established (Pusztai et al., 1982; Lorenzsonn and Olson, 1982; King et al., 1980a and b; Wilson et al., 1980). In addition to its toxicity, this glycoprotein is particularly stable to thermal treatment (Coffey et al., 1985) and therefore is of concern if the beans are not sufficiently cooked, as is sometimes the case when prepared at high elevations or in crock-pot cookers.

The toxicity of dry beans is well recognized. Noah et al. (1980) reported seven outbreaks of human food poisoning in Britain that were attributed to undercooked kidney beans. Experimental animals are also sensitive to raw dry beans. Jaffe (1960) was the first to demonstrate the oral and parenteral toxicity of PHA to rats. Since that time, there have been a number of reports concerning the toxicity of a variety of dry beans or their lectins (Kakade and Evans, 1965; Kakade and Evans, 1966; Evans and Bandemer, 1967; Hewitt et al., 1973; Jayne-Williams and Burgess, 1974; Rattray et al., 1974). It is now agreed that the most toxic agents in dry beans are the lectins (Honavar et al., 1962; Jaffe and Vega-Lette, 1968; Liener, 1976; King et al., 1980a and b; Wilson et al., 1980; Pusztai et al., 1981; King et al., 1982;

Lorenzsonn and Olsen, 1982). The concentration of lectin in beans is correlated to the amount of hemagglutinating activity and red kidney beans have the most potent hemagglutinating activity of the P. vulgaris varieties tested (Jaffe and Vega-Lette, 1968).

Since PHA is a glycoprotein with carbohydrate-binding capabiliity, affinity chromatography can be used for purification. Leavitt et al. (1977) successfully used thyroglobulin-Sepharose and Ohtani et al. (1980) used concanavalin A-Sepharose to produce purified PHA. According to Yachnin and Svenson (1972), Miller et al. (1973) and Felsted et al. (1976), PHA is most probably a family of five tetrameric proteins composed of only two different subunits, a potent erythrocyte agglutinator (E subunit) and a lymphocyte stimulator (L subunit).

This paper reports the results of an investigation of the stability of the hemagglutinating activity of purified PHA (PHA-P) to thermal, chemical and enzymatic abuse.

Materials and Methods

PHA Purification

Ammonium Sulphate Precipitation. Concentrated lectin fractions were precipitated from bean extracts by the method of Jaffe and Hannig (1965). Dark red kidney beans (Michigan Foundation Seed, East Lansing, MI) were used as the source of PHA in this study. One kg of hammermilled bean flour (sieve size # 50) was extracted overnight at 4^{o}C with 5 L of physiological buffered saline. Crude PHA was precipitated from the extract by the addition of ammonium sulphate to 75 % saturation. The precipitate was collected by centrifugation,

resuspended in distilled water and precipitated again with 75 % Ammonium Sulphate. The precipitate was dialyzed against several changes of distilled water at 4°C, freeze dried and stored frozen (-3°C) for later use.

Affinity Chromatography. Phytohemagglutinin (PHA) was purified by concanavalin A-agarose affinity chromatography. For purification, crude PHA was added to con A-agarose (Sigma Chemical Co., St. Louis, MO) and allowed to stand for one hour. The non bound protein was removed from the columns by washing with distilled water and saline at pH 7.2 until absorbance at 280 nm had stabilized. Removal of the bound PHA was accomplished by elution with PBS containing α-methyl-D-mannoside. The sugar was removed from the gel by elution with pH 3.5 Acetate buffer containing 2 M sodium chloride. The ultraviolet absorbance of the eluant was monitored using an ISCO model UA-2 Ultraviolet Analyzer (Instrumentation Specialties Co Inc., Lincoln, NE).

Thermal Inactivation

Purified PHA (PHA-P) was diluted with PBS (5 mg/mL) and 0.5 mL aliquots were introduced to 6 x 50 mm test tubes. The tubes were placed in either a water bath or oil bath and exposed to the following time/temperature conditions: 2, 4 and 8 hours at 70°C; 2, 4 and 8 hours at 75°C; 2, 4 and 8 hours at 80°C; 2, 4 and 8 hours at 85°C; 1, 2 and 4 hours at 90°C; 15, 30, 45, 60 and 75 minutes at 95°C; and 5, 10, 15, 20, 25 and 30 minutes at 100°C. The samples were withdrawn after treatment, cooled immediately in an ice water bath to 0°C and stored frozen (-3°C) until the hemagglutinating activity and electrophoretic analyses were performed. Three replicates of this experiment were performed.

Chemical Treatment

PHA-P was again diluted with PBS (5 mg/mL) and 2 mL aliquots were placed in 12 x 175 mm test tubes. The PHA-P was exposed to the following chemical agents: 2 M NaCl; 5 M urea and 5 % mercaptoethanol. In addition to these treatments, the PHA-P was dissolved in PBS adjusted to either pH 12 or pH 3. The PHA-P was held in treatment for times of 30, 60 and 120 minutes and the electrophoretic analyses and the hemagglutinating activity determined immediately following incubation. Three replicates of this experiment were performed.

Enzymatic Treatment

PHA-P was diluted with PBS (5 mg/mL) and 2 mL aliquots were placed in 12 x 175 mm test tubes. The following enzymes (all from Sigma Chemical Co., St. Louis, MO) were added to the PHA at a level of 0.05 mg/mL: pepsin (3200 units/mg protein); trypsin (14,600 BAEE units/mg protein); chymotrypsin (58 units/mg protein); peptidase (100 units/g solid); protease (6.0 units/g solid); pancreatin (4 x NF grade); alanine amino-peptidase (6.5 units/mg protein); α-amylase (1900 units/mg protein); β-amylase (850 units/mg protein); mannosidase (17 units/mg protein); and neuraminidase (19 units/mg protein). In each case the PHA was incubated with the enzyme for one, two and three hours and the digestion mixture assayed for hemagglutinating activity and subjected to electrophoretic analysis immediately. Three replicates of this experiment were performed.

Hemagglutinating Activity

The hemagglutinating activity of the purified and treated lectin was determined using the cell counting method of Coffey et al. (1985). These experiments were conducted in triplicate with duplicate

determinations of hemagglutinating activity made on each sample.

Electrophoretic Separations

Discontinuous Polyacrylamide Gel Electrophoresis (DISC-PAGE). DISC-PAGE was used to resolve the component peptide patterns of the treated PHA-P. The method used was that of Davis (1964) but staining was accomplished with 0.04 % Coomassie Brilliant Blue G-250 in 3.5 % perchloric acid overnight. For all DISC-PAGE evaluations, 11 % acrylamide gel concentration was used for the running gel and the proteins were subjected to 1 mA/tube for 10 minutes followed by 3 mA/tube for the remainder of the separation. The gels were destained and stored in 7 % aqeous acetic acid.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was performed to resolve the component peptides of the treated samples according to their molecular weight. The method used was that of Weber and Osborne (1969). For all SDS-PAGE evaluations, 10 % acrylamide gel concentration was used and the gel tubes were allowed to polymerize for 24 hours before use. The tubes were subjected to 3 mA/tube for 10 minutes followed by 8 mA/tube for the remainder of the separation. After development, the gels were stored in 7 % acetic acid. A series of molecular weight standards were run along with the bean extracts and treated PHA-P. The protein standards were obtained from Sigma Chemical Co. (St. Louis, MO) used and their molecular weights in daltons were: Phosphorylase B (94,000); Bovin serum albumin (67,000); Ovalbumin (43,000); Carbonic anhydrase (30,000); Soybean trypsin inhibitor (20,000); and α-Lactalbumin (14,400).

Results and Discussion

Purification of PHA

Purified phytohemagglutinin (PHA-P) was resolved from saline kidney bean extracts by chromatography on concanavalin A-Sepharose. Following addition of the bean extract, the column was washed with PBS until the absorbance at 280 nm had stabilized. Figure 10 illustrates that a substance with absorbance at 280 nm eluted following incorporation of 0.01 M ∝-methyl-D mannoside. This fraction was collected and found to have a high hemagglutinating activity. Several cycles of column charging, elution and regeneration were performed and this same fraction was collected in each cycle, dialyzed and freeze-dried. The tan powder produced was found to contain 87 % protein as determined by micro-kjeldahl analysis (N x 6.25). This protein concentration, although low, is consistent with the fact that PHA is a glycoprotein with 7.8 to 7.9 % carbohydrate (Allen et al., 1969; Ohtani et al., 1980).

The protein purified by affinity chromatography was subjected to DISC gel and SDS gel electrophoresis and compared to a standard PHA. The patterns developed by the electrophoresed protein (PHA-P) were essentially identical to the pattern of the standard PHA. The results of the electrophoresis are shown in Figure 11. Based on the electrophoretic patterns and the high hemagglutinating activity data, this protein was designated as a purified phytohemagglutinin.

Hemagglutinating Activity

Thermal Treatment. The diluted PHA-P was subjected to thermal stress, the hemagglutinating activity determined and the abused protein subjected to electrophoretic analysis. PHA-P is resistant to activity

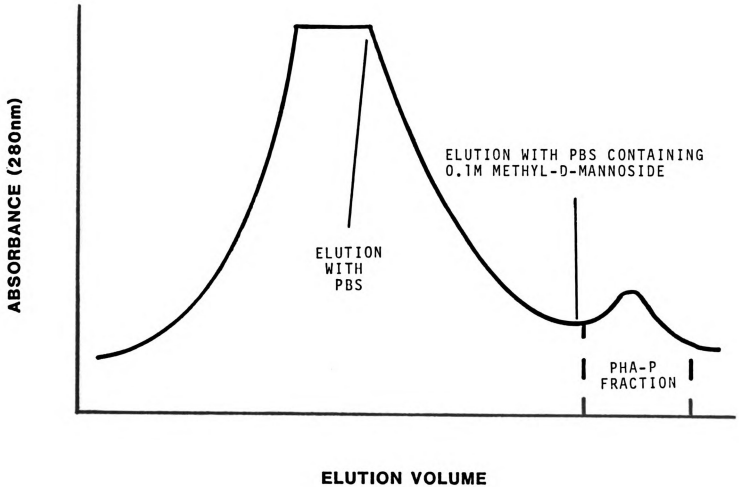
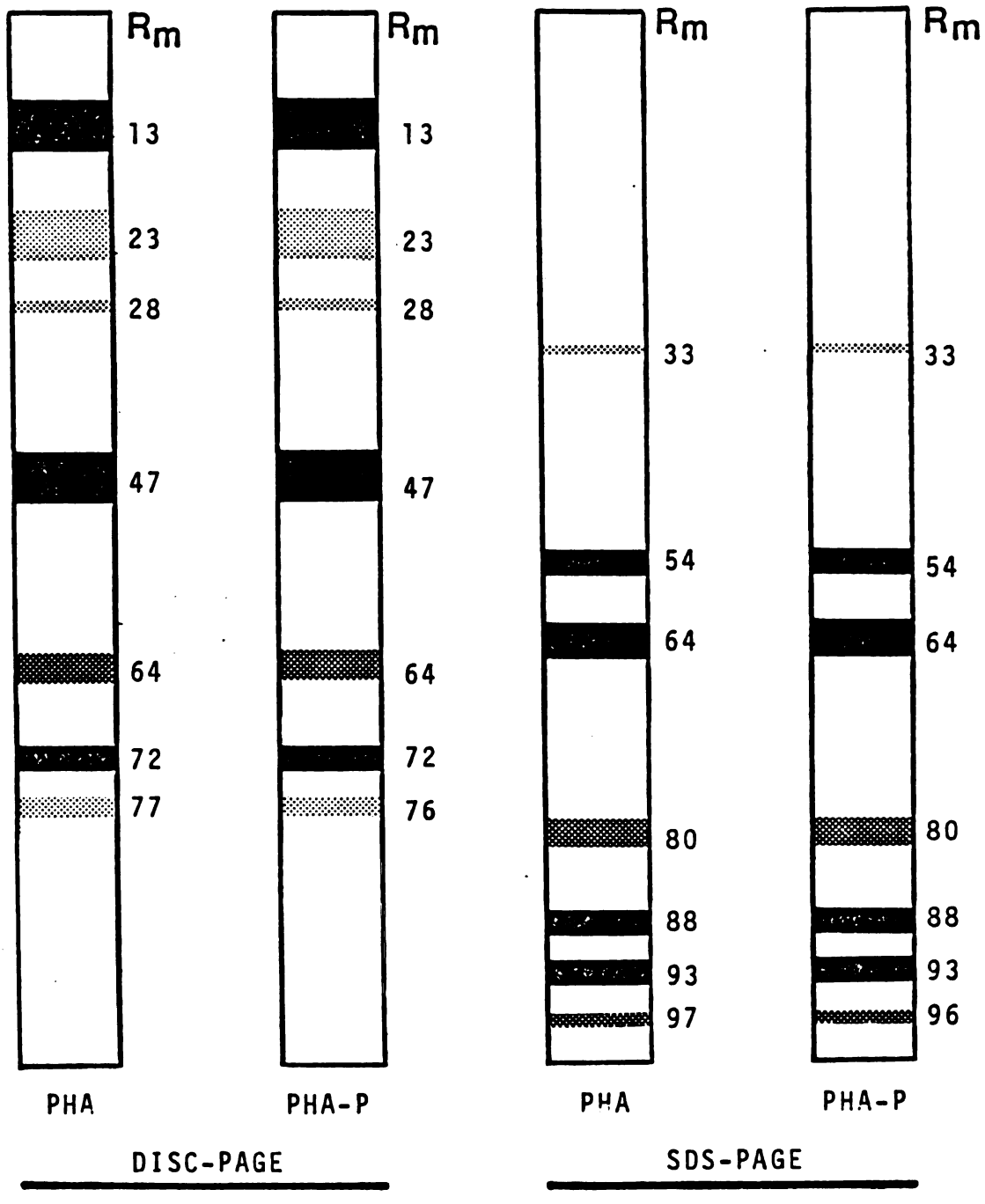


Figure 10. Elution of PHA-P from concanavalin A-agarose affinity chromatography gel



(R_m = relative mobility x 100)

Figure 11. Electrophoretic analysis of PHA and PHA-P

loss due to freezing. Figure 12 shows the hemagglutinating activity of 0 to 250 ug of PHA-P at different times during frozen storage. No dramatic change was found in the hemagglutinating activity of the PHA-P over a seven month period of frozen storage. Table 2 summarizes the statistical treatment of the data for this experiment. A comparision of the main effect of time on hemagglutinating activity with the LSD ($P<0.01$) value shows no significant differences for this treatment. In agreement with this comparison, an investigation of the interaction between time and levels of PHA-P show no significant differences between samples within a level. Therefore no significant differences were detected in hemagglutinating activity of freeze-dried PHA-P stored for seven months at -3°C.

The heat inactivation of PHA-P is shown in Figure 13. This figure represents the retention in % PHA activity for treatments at 70°, 80°, 90° and 100°C. Heating at temperatures of 80°C or greater resulted in rapid inactivation to 6 % or less of native PHA-P activity. Holding at 70°C resulted in the following activity retention figures: 2 h/82.67 %; 4 h/63.19 %; 6 h/41.25 %; and 8 h/24.17 %. The correlation coefficient of these data is -0.999 and such linearity between time and temperature indicates a first order reaction for the thermal inactivation of PHA-P. The regression equation for the 70°C inactivation data is:

% PHA Activity Remaining = 102.18 - 9.87(hours at 70°C)

The analysis of variance of this data (Table 3) shows there are significant differences ($P<0.01$) for temperature, time and the interaction between temperature and time. Of greatest interest are the differences between times at 70°C. A comparison of the values with the LSD ($P<0.01$) value show that the changes in hemagglutinating activity at

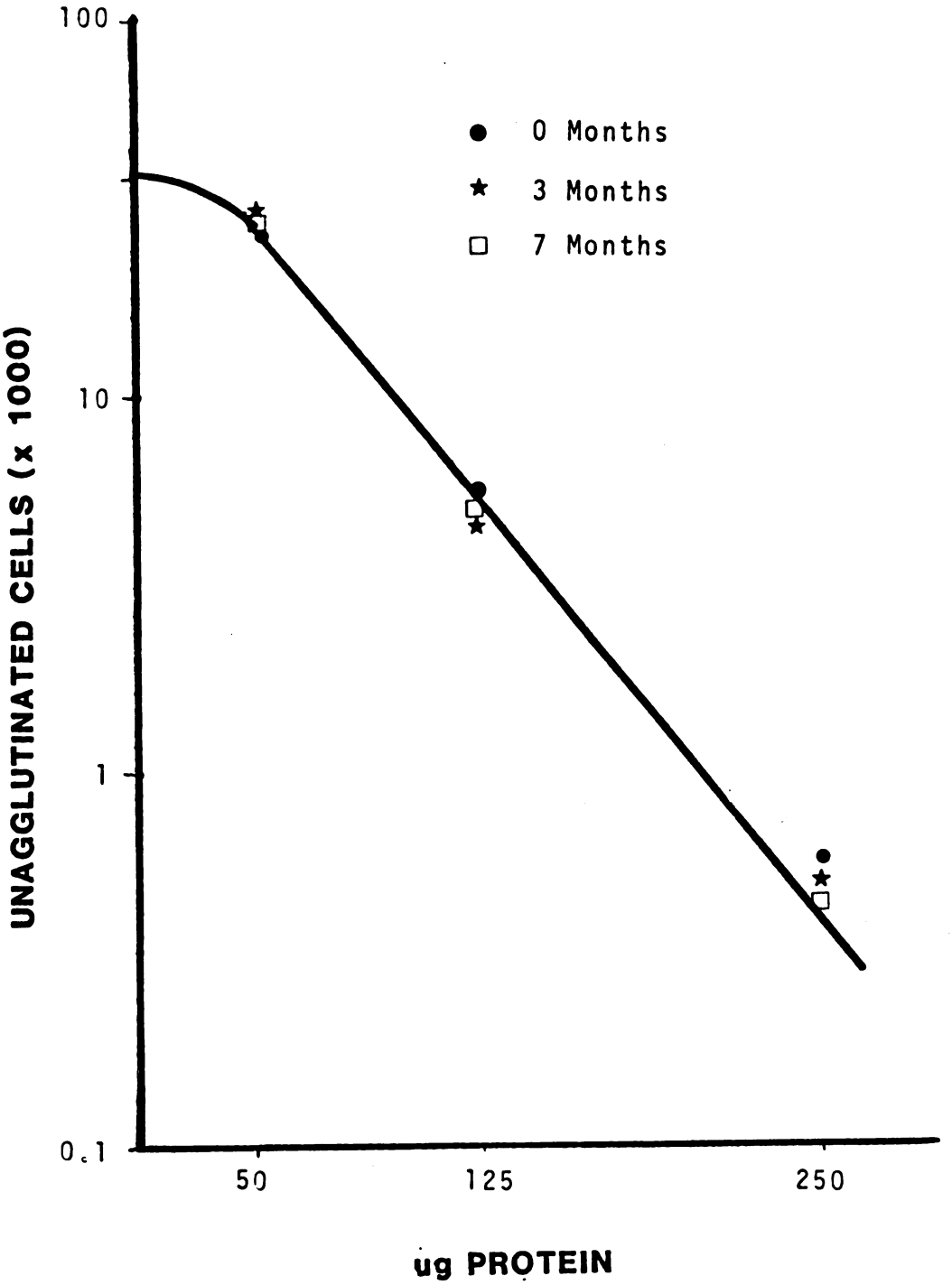


Figure 12. Effect of frozen storage (-3°C) on the hemagglutinating activity of PHA-P

Table 2. Statistical summary of effect of frozen storage on the hemagglutinating activity of PHA-P as measured by number of unagglutinated cells

MAIN EFFECT MEANS

TIME (MONTHS)	0	3	7
	11136	12397	11793

INTERACTION MEANS

	LEVEL (ug)		
TIME (months)	250	125	50
0	27500	5333	575
3	31933	4500	760
7	29500	5033	847

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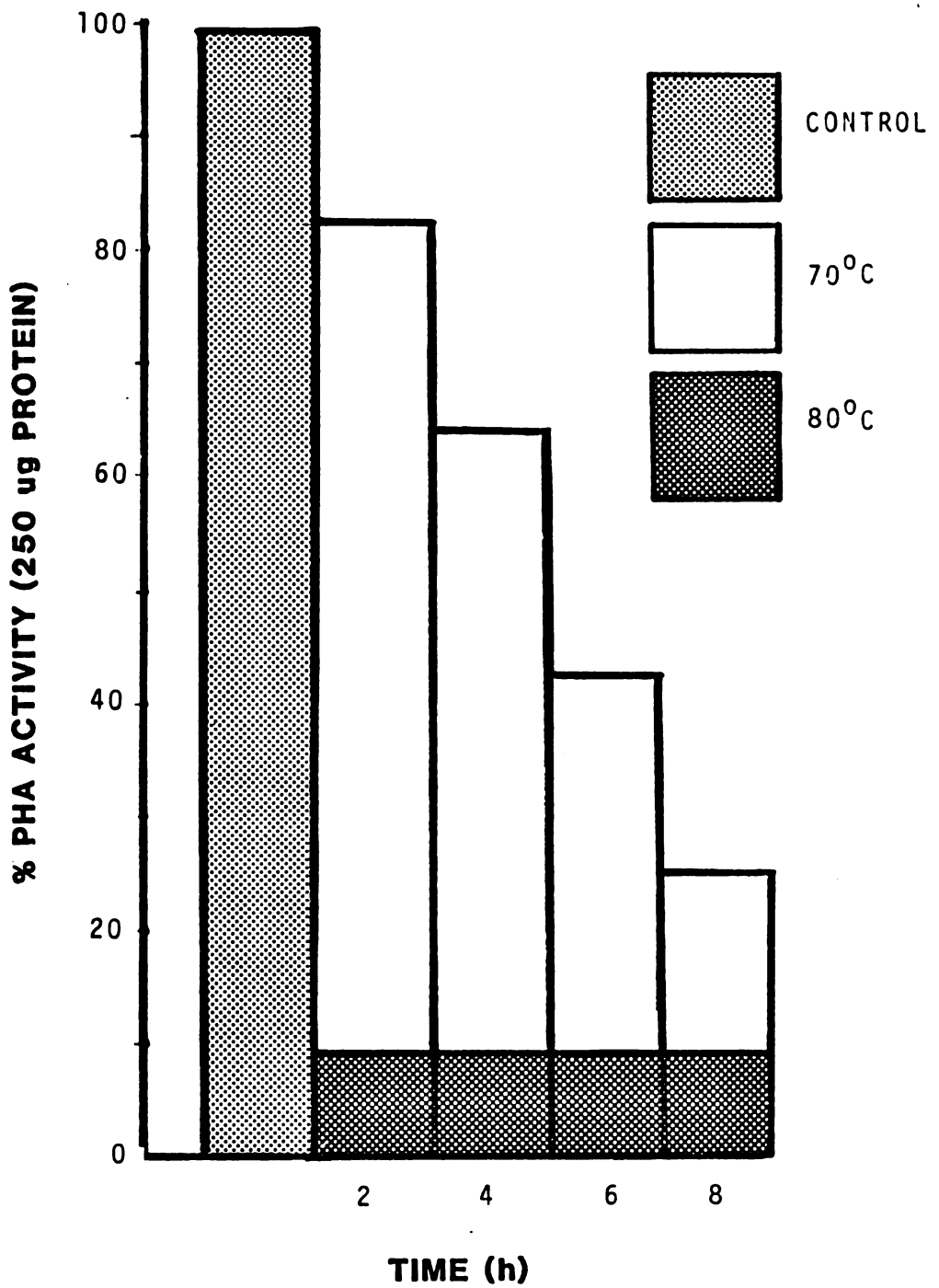
ANALYSIS OF VARIANCE OF EFFECT OF FROZEN STORAGE ON HEMAGGLUTINATING ACTIVITY OF PHA-P

SOURCE	degrees of freedom	mean squares
TIME	2	3,580,000.00**
LEVEL	2	2,200,000,000.00**
TIME X LEVEL	4	5,900,000.00**
ERROR	18	827,623.00
TOTAL	26	171,000,000.00

** Significant at the 1 % level



(R = -0.999 @ 70°C; % Activity = 102.18 - 9.87 (h at 70°C))

Figure 13. Effect of thermal treatment on hemagglutinating activity of PHA-P

Table 3. Statistical summary of effect of thermal treatment on the hemagglutinating activity of PHA-P as measured by the % PHA activity retention

MAIN EFFECT MEANS

TEMPERATURE (^{o}C)	
70	80
52.82	9.27

TIME (h)			
2	4	6	8
46.33	39.13	22.29	16.46

INTERACTION MEANS

	TIME (h)			
TEMP (^{o}C)	2	4	6	8
70	82.67	63.19	41.25	24.17
80	10.00	15.00	3.33	8.75

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ANALYSIS OF VARIANCE OF THERMAL TREATMENT ON THE HEMAGGLUTINATING ACTIVITY OF PHA-P

SOURCE	degrees of freedom	mean squares
TEMPERATURE	1	11,378.74**
TIME	3	1175.91**
TEMPERATURE X TIME	3	846.28**
ERROR	16	22.25
TOTAL	23	773.97

** Significant at the 1 % level

each time tested were significantly different.

These results are consistent with those reported by Aw and Swanson (1985) indicating inactivation of dry bean lectin at temperatures of 70°C or greater. These results and those of the first study suggest that there is a protective effect afforded by the seed cotyledon during cooking and it is not simply a heat transfer lag which is responsible for greater stability in the intact beans. The temperatures needed for inactivation of hemagglutinating activity of whole beans are much higher than those for the PHA-P.

Chemical Treatment. Figure 14 shows the effect of a series of selected chemical conditions on the hemagglutinating activity of PHA-P. Following a two hour incubation treatment in the chemically controlled PBS media, the following % PHA activities were determined: 2 M sodium chloride/93.3 %; pH 3 (hydrochloric acid)/81 %; 5 % mercaptoethanol/76 %; 5 M urea/61 %; and pH 12 (sodium hydroxide)/35 %. These results conform to expectations regarding PHA stability. Sodium chloride had a slight but insignificant effect on hemagglutinating activity and this is expected as PHA is a salt-soluble glycoprotein and has maximum effectiveness at salt concentrations of approximately 0.85 %. There was also a slight reduction in hemagglutinating activity when PHA-P was held at pH 3.0 for up to two hours. In this case, the reduction in pH was probably not drastic enough to hydrolyze sufficient peptide bonds of the protein to make a large impact on the hemagglutinating activity.

Mercaptoethanol is a reducing agent widely used in protein research as it reduces the disulfide bridge between peptide chains. Mercaptoethanol caused a 24 % reduction in PHA-P hemagglutinating

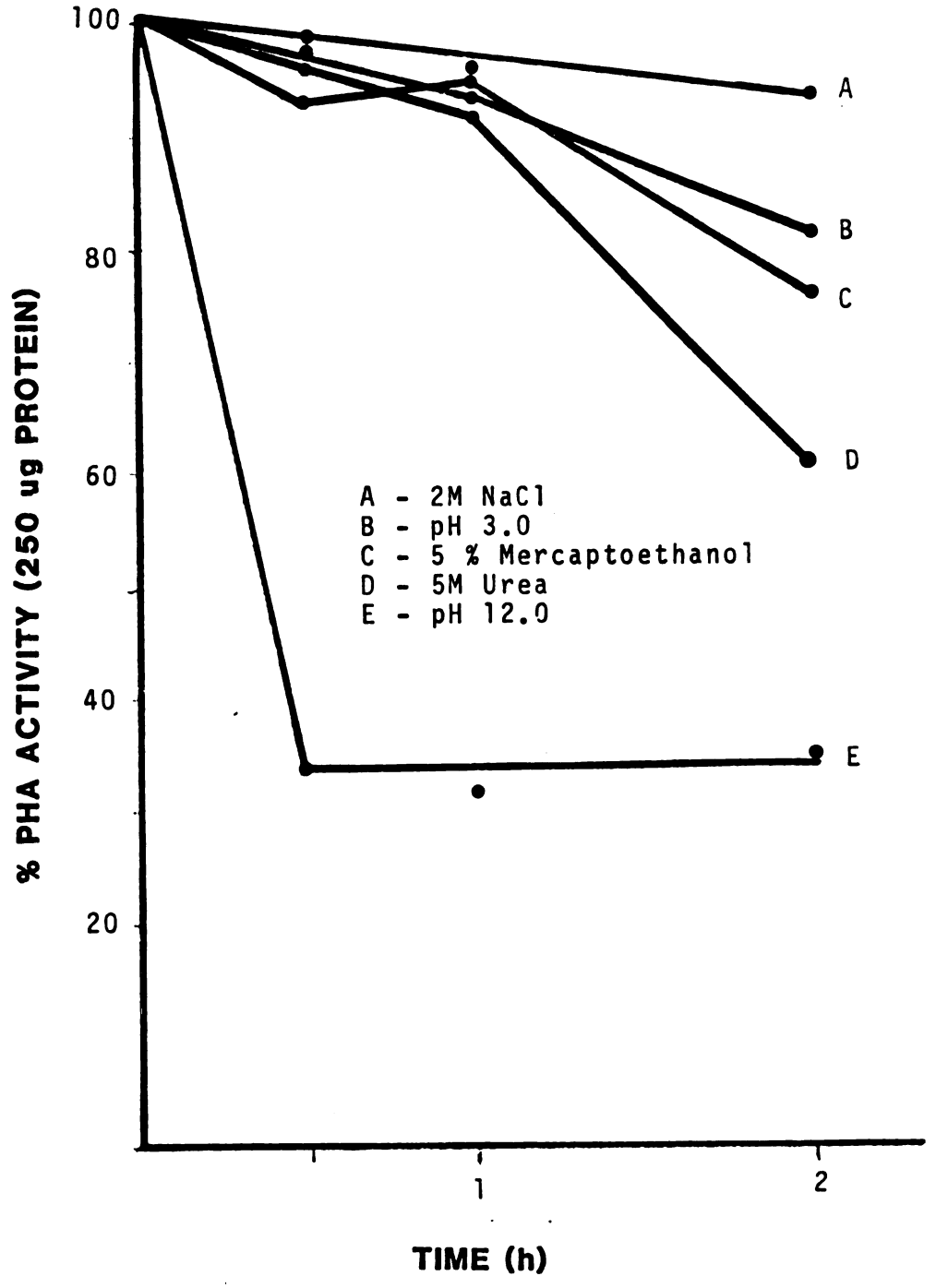

Figure 14. Effect of chemical agents on the hemagglutinating activity of PHA-P

activity. This relatively low depression of PHA activity may be attributed to the structure of the protein since there are no disufide bonds between the four subunits of PHA. Further, the quaternary structure is not an absolute requirement for hemagglutinating activity as Felsted et al. (1977 and 1981) reported that the purified subunits retain much of their agglutinating ability.

Urea is a particularly effective denaturation agent as it destabilizes the quatenary structure of multi-subunit proteins and causes an unfolding of the protein chains. Urea caused a reduction in hemagglutinating activity of nearly 40 % in just two hours. Since urea causes severe changes in a protein chain, one might expect that the reduction in activity would be greater than 39 %. One explanation for the results would be that the urea concentration was seriously reduced when the treated PHA-P sample was introduced to the blood cell suspension, thus allowing the PHA-P to partially return to its original conformation. This may explain why the results of 0.5 and 1 hour are not significantly different. Perhaps the degree of unfolding in both of these instances was less than could be reconformed by dilution in the assay procedure. The degree of unfolding by two hours however, may have been too great to be alleviated by dilution.

The results of treatment at pH 12.0 are dramatic. From Figure 14, it is obvious that treatment of PHA-P by holding it at high pH is effective in causing a large reduction in hemagglutinating activity. Incubation of PHA-P at pH 12 resulted in a 65 % reduction in hemagglutinating activity, much greater than any other chemical treatment.

Table 4 presents the results of the analysis of variance of the

Table 4. Statistical summary of effect of chemical treatment on the hemagglutinating activity of PHA-P as measured by the % PHA activity retention

MAIN EFFECT MEANS

CHEMICAL TREATMENT				
SALT	pH 3.0	M.E.	UREA	pH 12.0
94.30	89.03	89.45	84.26	38.79

INTERACTION MEANS

	TIME (minutes)		
CHEM. TREATMENT	30	60	120
SALT	96.33	94.69	91.89
pH 3.0	91.94	94.35	80.79
M.E.	95.96	94.67	77.73
UREA	95.94	91.96	64.88
pH 12.0	36.58	36.79	43.00

M.E. = MERCAPTOETHANOL

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ANALYSIS OF VARIANCE OF CHEMICAL TREATMENT ON THE HEMAGGLUTINATING ACTIVITY OF PHA-P.

SOURCE	degrees of freedom	mean squares
CHEMICAL	4	4698.53**
TIME	2	637.14**
CHEMICAL X TIME	8	185.58**
ERROR	30	13.08
TOTAL	44	498.76

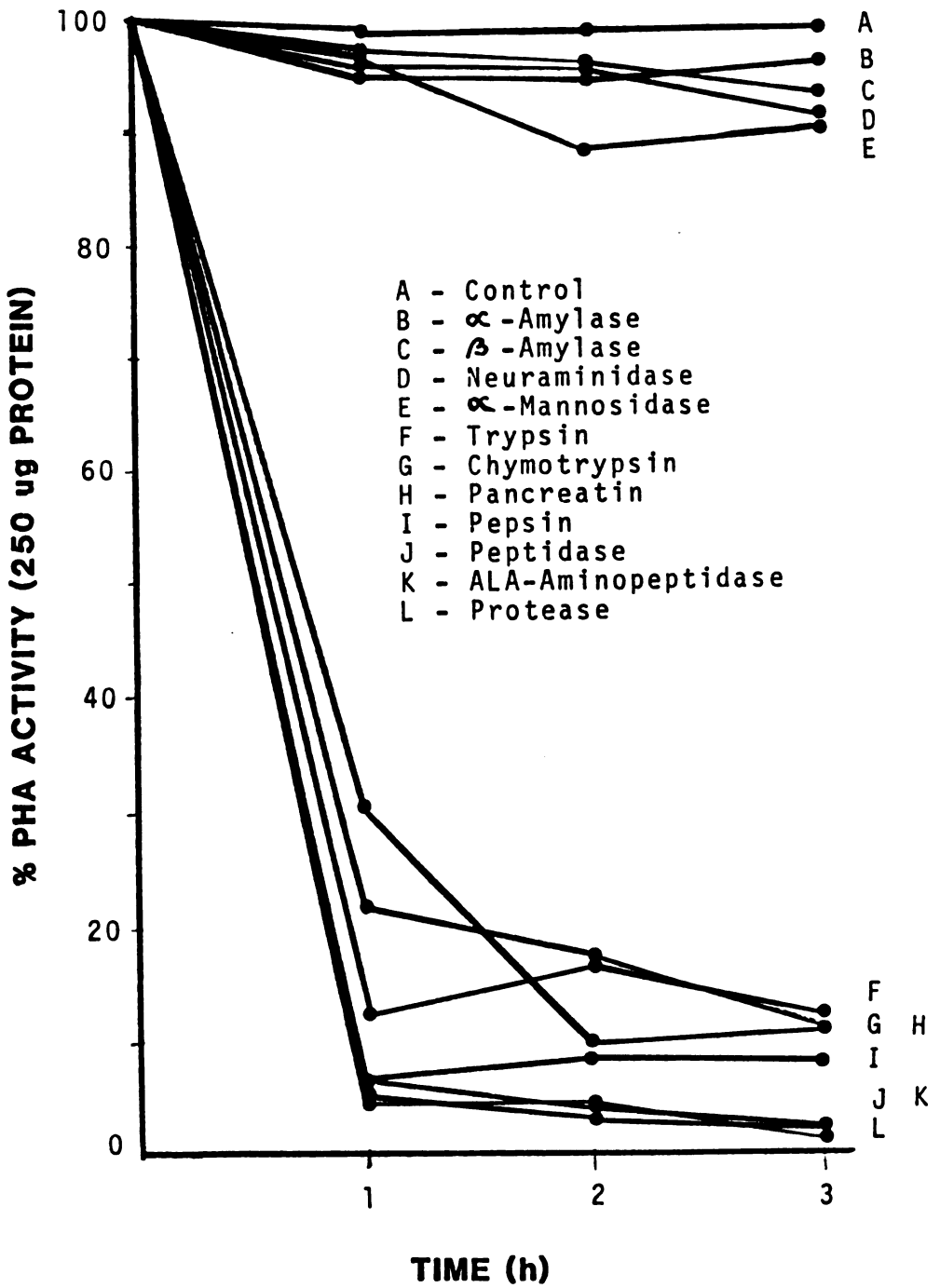
** Significant at the 1 % level

effect of chemical treatment on PHA-P. The cell means for the main effect of chemicals show that all treatments are significantly different ($P<0.01$) except for the pH 3.0 and 2 M sodium chloride treatments. A comparison of the cell means for the interaction of treatment with time show all treatments were significantly different ($P<0.01$) except for pH 3.0 and sodium chloride which were not significantly different from each other.

These results indicate that use of alkaline cookery conditions may be a practical approach to enhance lectin inactivation, especially for rural emerging countries. Treatment of corn by lye is already a well established practice for the improvement of its nutritional value. If a similar improvement in the nutritive value of dry beans could be accomplished by soaking in high pH media, it may represent a substantial improvement of protein quality in developing countries.

Enzymatic Treatment. The results of enzymatic treatments of PHA-P are summarized in Figure 15 and Table 5. These data show that all proteases investigated resulted in a significant destruction of the hemagglutinating activity of PHA-P with time. For all proteolytic enzymes used, two hours of incubation were sufficient to cause a reduction of 88 - 98.5 % of the native PHA-P activity.

These data also show clearly that there is a slight but insignificant reduction in hemagglutinating activity after incubation of PHA-P with α-amylase and β-amylase. From structural considerations, it was expected that the amylases would have little effect on the hemagglutinating activity. However, these were investigated because of a potential to incorporate high amylase activity in bean soaking medium and thus provide improvement in softening and lectin inactivation.



Enzyme level = 1 % substrate level (0.05 mg/mL)

Figure 15. Effect of enzymatic digestion on the hemagglutinating activity of PHA-P

Table 5. Statistical summary of effect of enzymatic treatment on the hemagglutinating activity of PHA-P as measured by the % PHA activity retention

	MAIN EFFECT MEANS	INTERACTION MEANS TIME (h) 1	2	3
ENZYME				
PEPSIN	7.45 b	6.18	8.21	7.96 a,b
TRYPSIN	13.67 a	12.25	16.53	12.17 a
CHYMOTR.	17.03 a	30.42	9.79	10.88 a,b
PROTEASE	3.47 b	4.50	4.42	1.49 b
PEPTIDASE	3.97 b	6.17	3.58	2.17 a,b
PANCREATIN	16.75 a	21.67	17.58	11.00 a,b
ALA-AM-PEP.	3.66 b	5.58	3.22	2.17 a,b
NEURAM.	94.93 c	95.79	96.17	91.23 c
α-AMYLASE	96.09 c	97.10	94.65	96.51 c
β-AMYLASE	94.68 c	95.19	95.36	93.50 c
MANNOSIDASE	91.69	96.58	88.36	90.14 c
CONTROLS	98.99 c	98.79	99.09	99.09 c

CHYMOTR. = CHYMOTRYPSIN
ALA-AM-PEP. = ALANINE AMINO PEPTIDASE
NEURAM. = NEURAMINIDASE
Means followed by like letters are not significantly different (P<0.01)

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ANALYSIS OF VARIANCE OF ENZYMATIC TREATMENT ON THE HEMAGGLUTINATING ACTIVITY OF PHA-P

SOURCE	degrees of freedom	mean squares
ENZYME	11	16,152.56**
TIME	2	246.14**
ENZYME X TIME	22	49.08**
ERROR	72	22.72
TOTAL	107	1690.52

** Significant at the 1 % level

Incubation with amylases produced no significant decreases in hemagglutinating activity in this study.

Sialic acid is the terminal carbohydrate in the oligosaccharide chain and because of its position, it might be expected to play a role in a recognition event involved with erythrocyte agglutination. Neuraminidase cleaves terminal sialic acids and it was expected that hydrolysis of these residues might cause a decrease in the hemagglutinating activity of PHA-P. A review of the cell means of the main and interaction effects (Table 5) show that there was a slight reduction in the activity but this was not significantly different ($P<0.01$) than the control.

Similarly, α-mannosidase cleaves internal mannose residues and would be expected to cause a significant change in the carbohydrate moiety of PHA. If the carbohydrate residue is important in binding, this would be expected to have a great impact on its binding and therefore hemagglutinating potential. The results of this study when examined by analysis of variance (Table 5) show that there was a slight (non significant ($P>0.01$), significant ($P>0.05$)) decrease in hemagglutinating activity of PHA-P treated with α-mannosidase for three hours.

These results of treatment with carbohydrate hydrolyzing enzymes indicate that the carbohydrate moiety is not the sole determinant of hemagglutinating activity for PHA-P. However, since treatment with mannosidase did result in a slight but significant reduction in activity at three hours, the oligosaccharide chain is necessary for full activity. These data do not agree with the results of Ohtani et al. (1980) who reported that hemagglutinating activity did not decrease

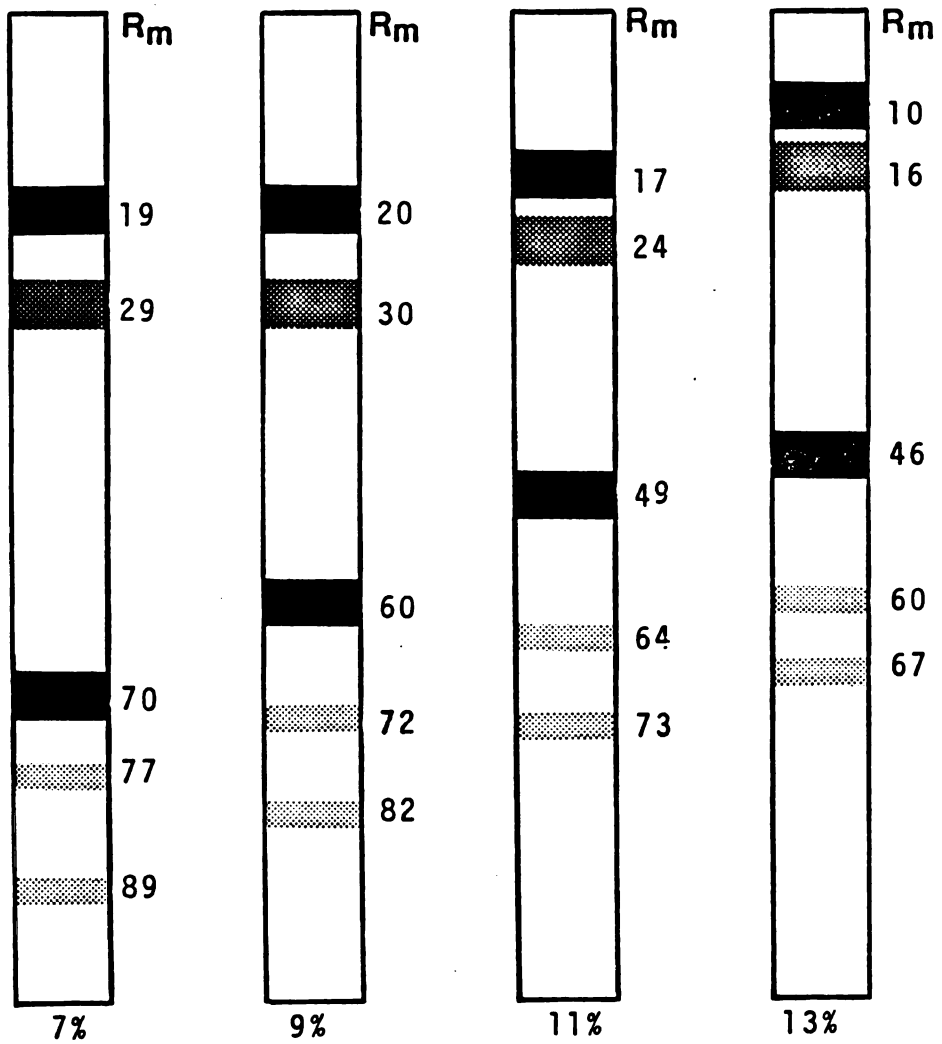
following digestion with α-mannosidase. However, their method of visual estimation of hemagglutinating activity probably was not sensitive enough to detect the difference.

Electrophoretic Analysis.

DISC-PAGE. The results of the DISC-PAGE evaluations are shown in Figures 16 through 19. Figure 16 shows the response of PHA to differing concentrations of acrylamide in the running gel. Based on the results, a concentration of 11 % was chosen for subsequent DISC-PAGE analyses in the study.

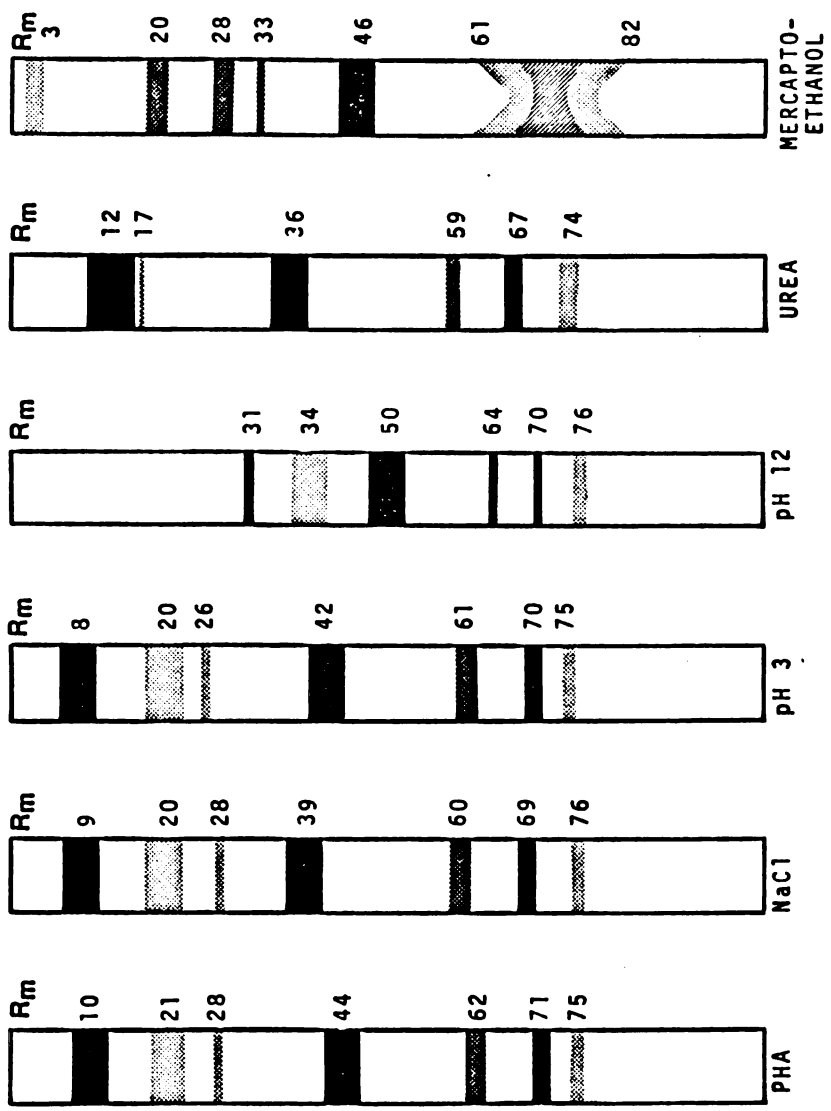
An evaluation of the PHA-P exposed to a variety of thermal treatments showed no dramatic changes in the protein patterns. It was expected that there may have been some visible changes due to thermal denaturation but this was not observed. The patterns of all the heated samples were essentially identical to the unheated control. The loss of hemagglutinating activity on heating is therefore probably due to unfolding of the peptide chains but not due to more severe destruction.

Exposure of the PHA-P to 2 M NaCl, pH 3.0 or 5 M urea has little effect on the gel patterns (Figure 17). Exposure to pH 12.0 however produced some changes. There was a decrease in the number of protein bands with R_m values less than 0.50. For bands with R_m of >0.60 there was no change. Incubation at pH 12.0 caused a shift in most of the bands although only slightly. This is probably due to the ionization of the amino acids at the high pH causing a shift in the charge/density ratio. Treatment of PHA-P with 5 % mercaptoethanol caused some change in the range of R_m 0.60 to 0.82. In this range there was a lack of resolution, however it seems that there was a concentration in the range



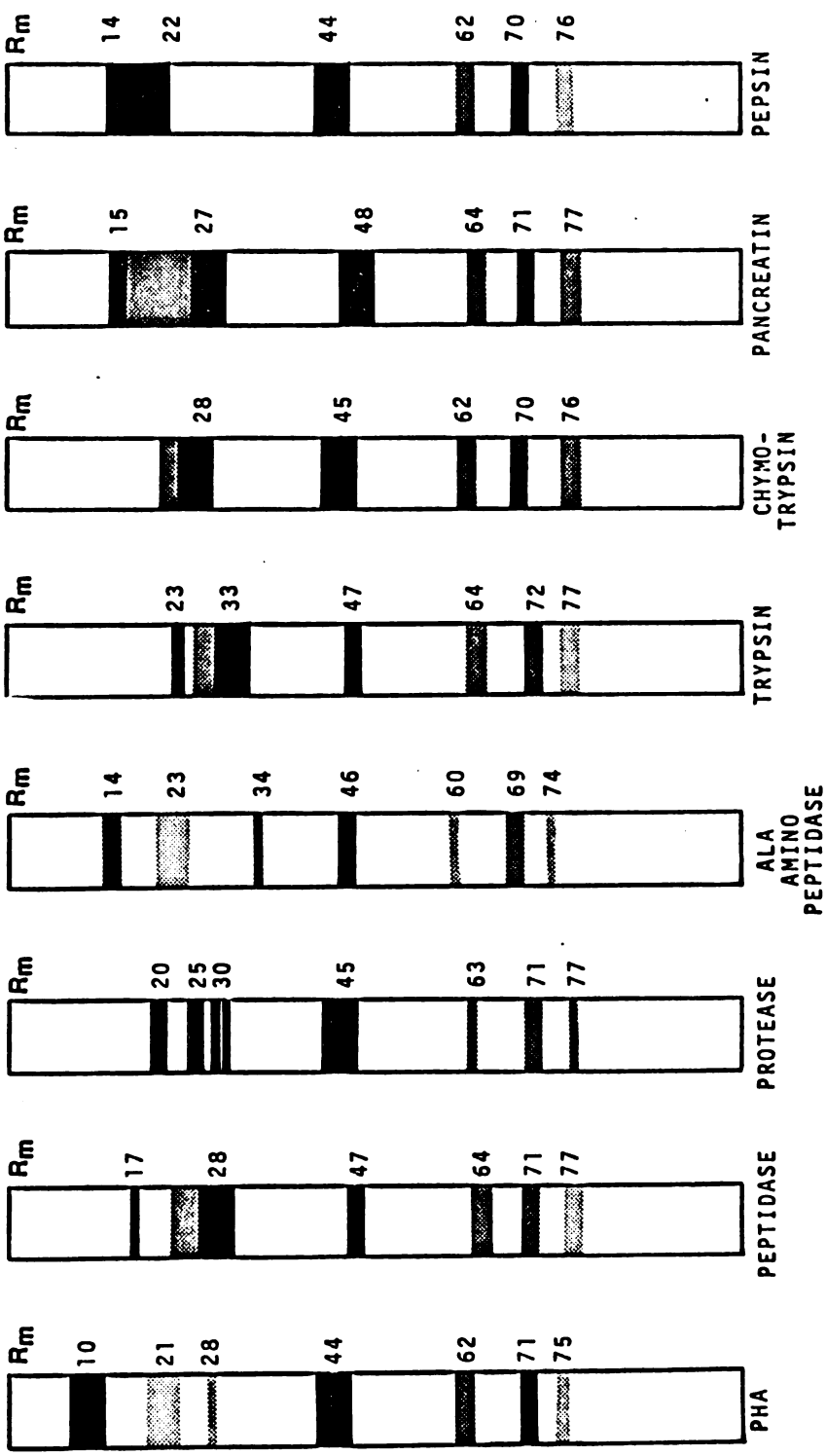
(R_m = relative mobility x 100)

Figure 16. DISC-PAGE analysis of PHA at varying acrylamide concentrations



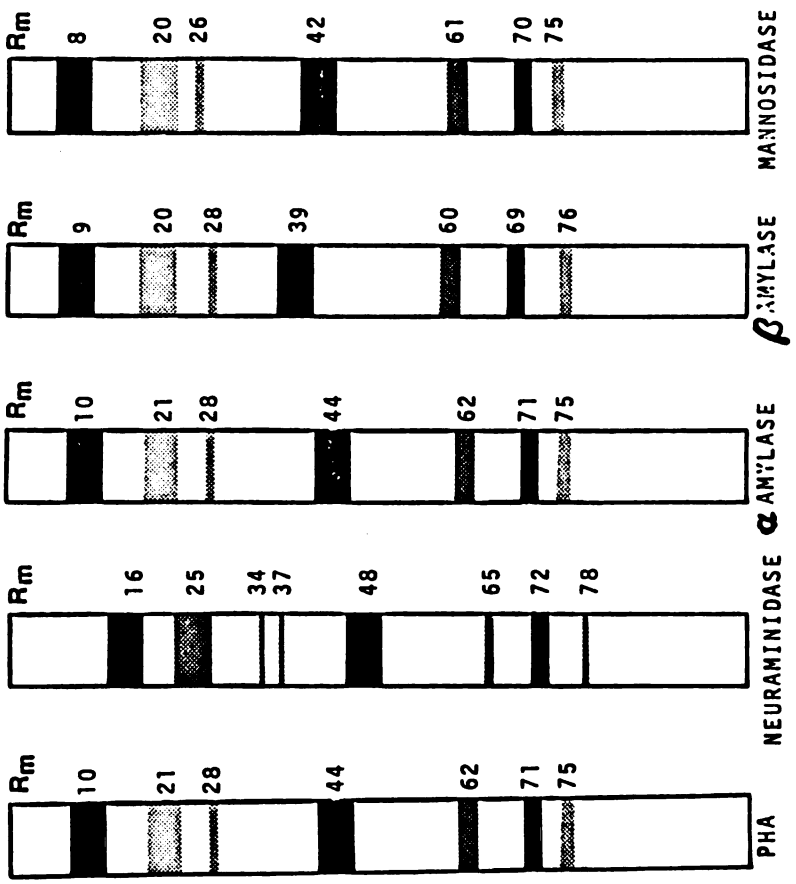
(R_m = relative mobility x 100)

Figure 17. DISC-PAGE analysis of PHA-P exposed to various chemical treatments



(R_m = relative mobility x 100)

Figure 18. DISC-PAGE analysis of PHA-P exposed to various proteolytic enzymes



(R_m = relative mobility x 100)

Figure 19. DISC-PAGE analysis of PHA-P exposed to various enzymatic treatments

of 0.7 and minor bands immediately above and below it. If it was just a case of poor resolution, it would agree with the results of the standard. However, this was observed in all three replicates.

Treatment of PHA-P with proteolytic enzymes (Figure 18) seemed to have little effect at R_m >0.44. There was a major band at 0.44 to 0.48 and a large band at 0.10 in the controls. All proteolytic enzymes were effective in reducing the presence of the 0.10 band and causing other minor changes at the lower R_m values. All of the carbohydrate hydrolyzing enzymes tested had essentially the same patterns as the control PHA-P (Figure 19). It is probable that the amylases had very little effect on the PHA-P structure. This is expected as there is no glucose in the PHA oligosaccharide chain. The effect of neuraminidase on PHA-P pattern is also negligible. It was expected that there may be a large effect on the gel pattern as there are terminal sialic acid residues in the oligosaccharide chain. However, the effect on the molecular weight after cleaving these residues is negligible and therefore was not observed in the pattern. The mannosidase treated PHA-P showed an increase in the R_m of the 0.50 band to 0.58. This is probably due to cleavage of the mannose residues in the chain, increasing the charge/density ratio of the residual peptide.

SDS-PAGE. The R_m values of the molecular weight standards are shown in Figure 20. According to these data, the major band occurred at R_m of approximately 0.60 which corresponds to a molecular weight of about 28,000 daltons. Felsted et al. (1981) reported that the molecular weight of the subunits of PHA were 31,700 and 29,900 for the E and L subunits respectively. Since SDS treatment denatures the native PHA-P prior to electrophoresis, it would be expected that the major band would

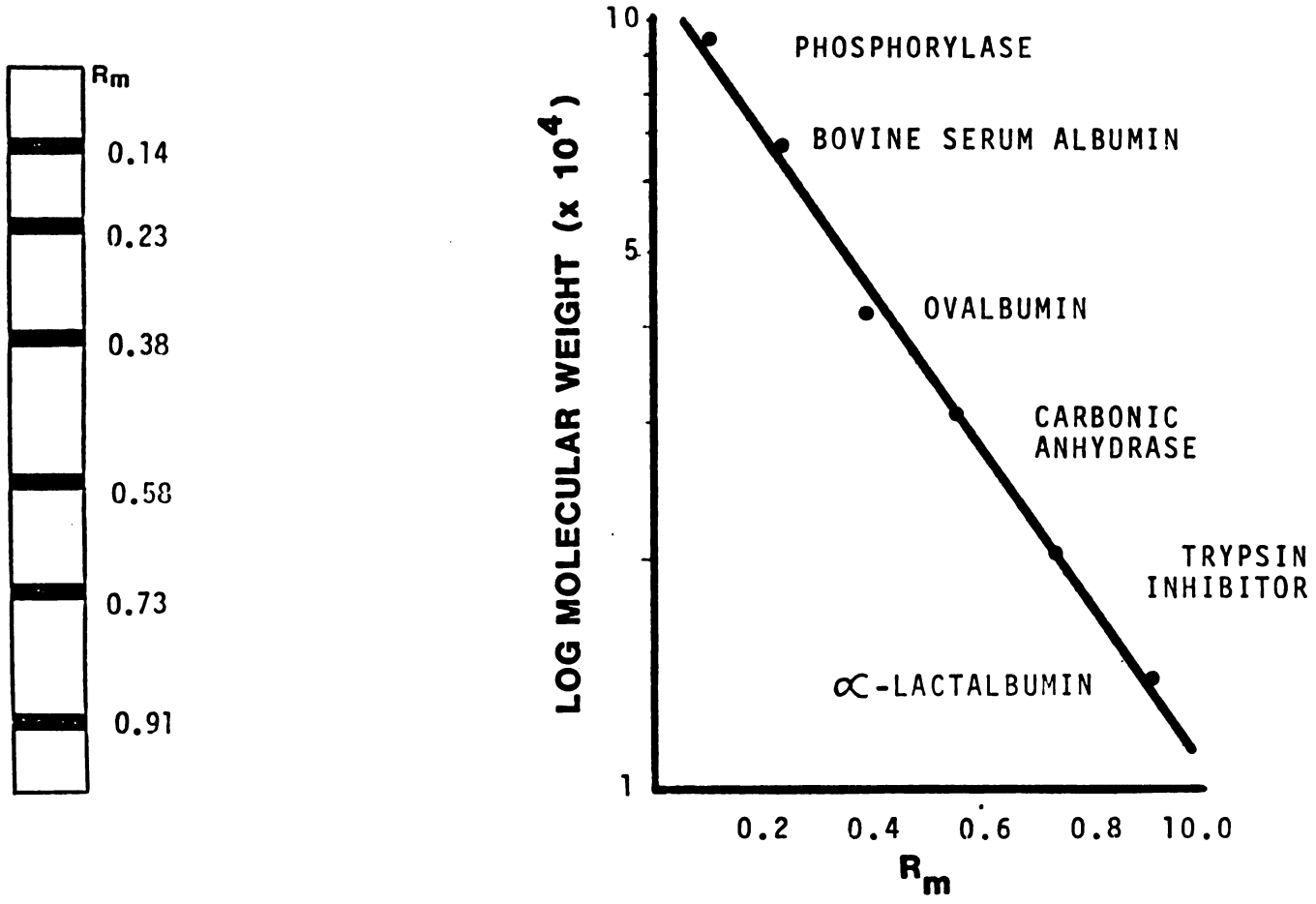


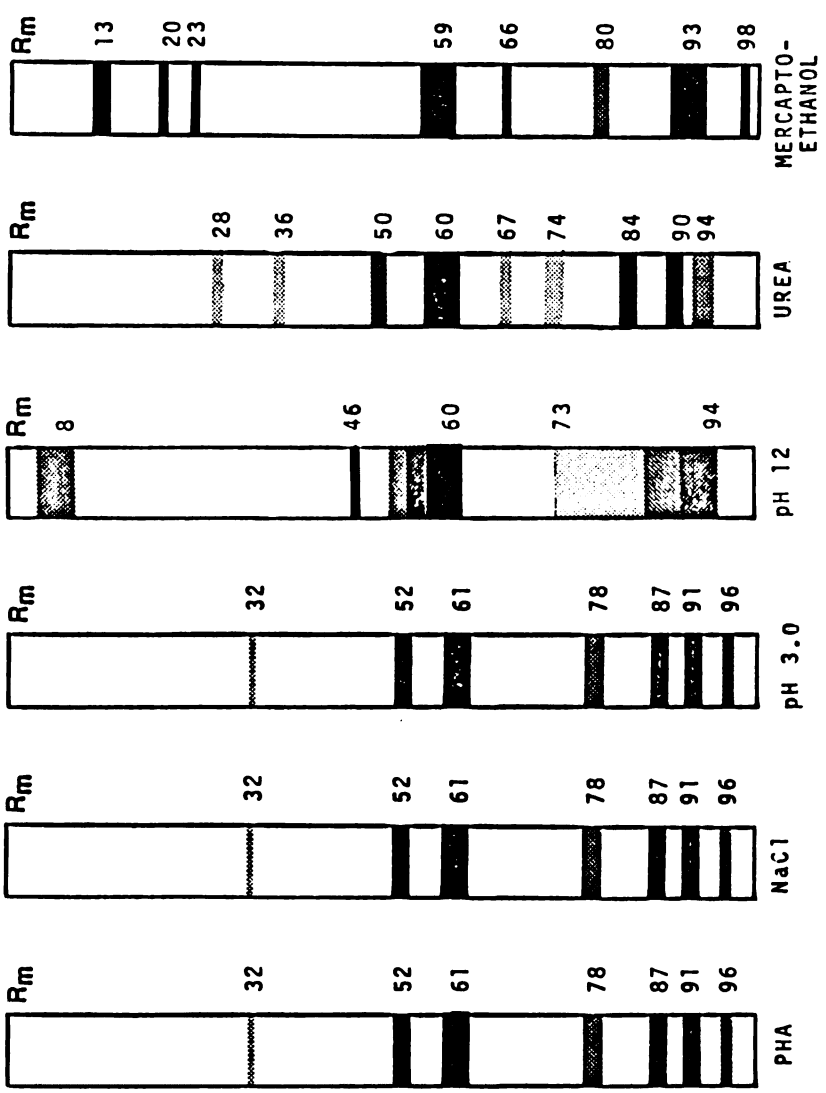
Figure 20. SDS-PAG electrophoresis of molecular weight standards (10 % acrylamide concentration)

be the average of the subunits. The major band observed at R_m = 0.60 in our study agrees well with the expected results for relative mobility.

As observed in the DISC-PAGE evaluations, there was no observable effect of thermal treatment or exposure to pH 3.0. These two treatments were indistinguishable from the control. Treatment at pH 12.0 did cause some changes in the elution profile (Figure 21). There was a lack of the 0.30 band and the presence of stained areas from 0.40 to 0.60 and from 0.73 to 0.94. This indicates an increase in the concentration of lower molecular weight species following this treatment. There was also a new band at 0.05 to 0.08 after treatment. This may be due to condensation of smaller proteins to produce larger species. Addition of urea had little effect also except for the presence of a new band at 0.67. Mercaptoethanol treatment produced gels similar to controls except that there were bands at 0.13, 0.20 and 0.23 following treatment.

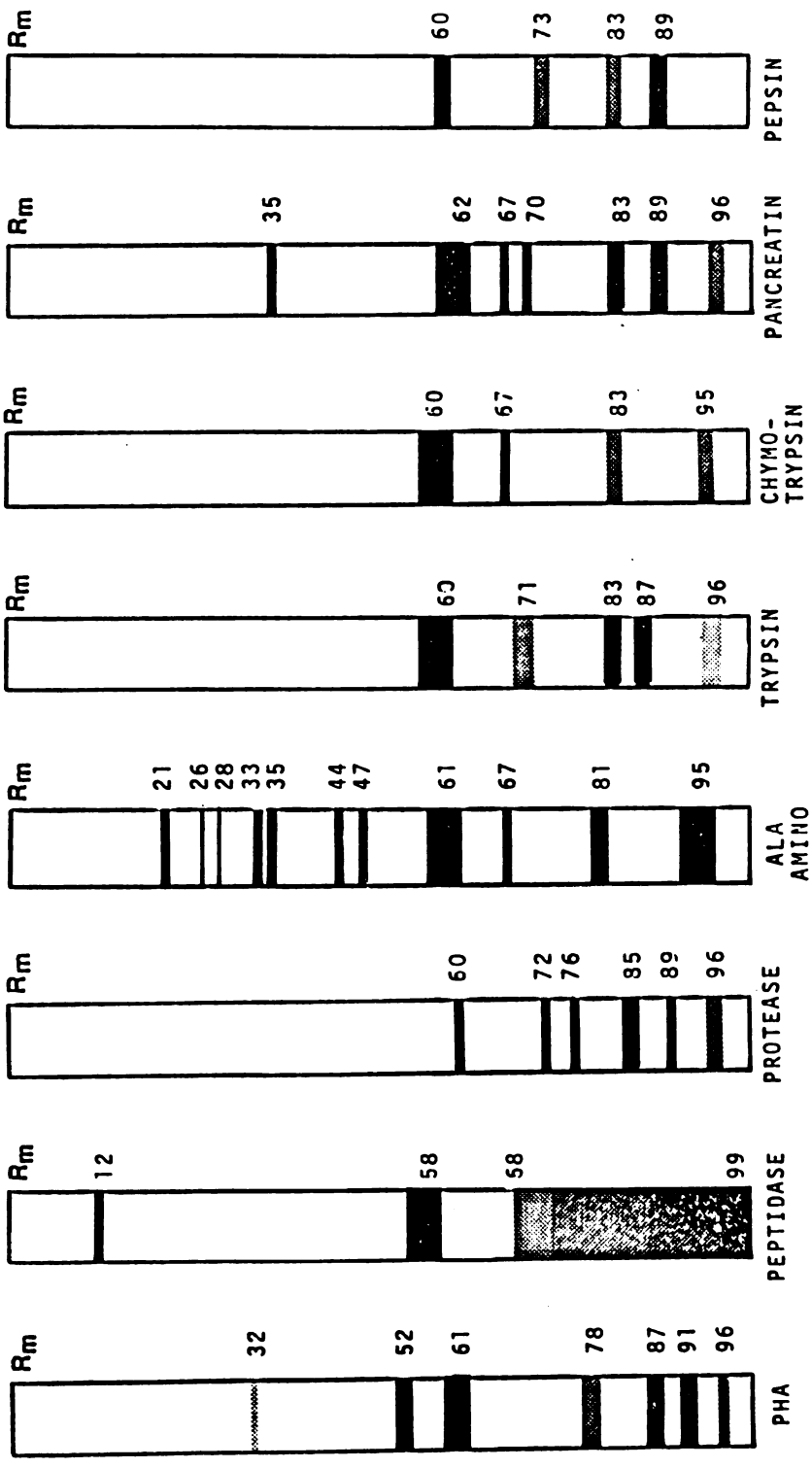
Digestion with proteolytic enzymes is characterized by a general lack of high molecular weight components in the gel patterns (Figures 22 and 23). Peptidase is unique in that it caused a broad diffuse staining from 0.68 to 1.0 along with the band at 0.58 and a minor band at 0.12. This indicates reduction in molecular weight of the component peptides. Alanine amino peptidase treatment caused an increase in the number of bands corresponding to higher molecular weight. Digestion with the other proteolytic enzymes yields similar patterns that lack higher molecular weight peptides.

Incubation of the PHA-P with amylases yields patterns that are essentially identical to that of the untreated PHA-P. This is expected for the same reason outlined before: there is a lack of glucose in the oligosaccharide chain. Treatment with α-mannosidase also resulted in a



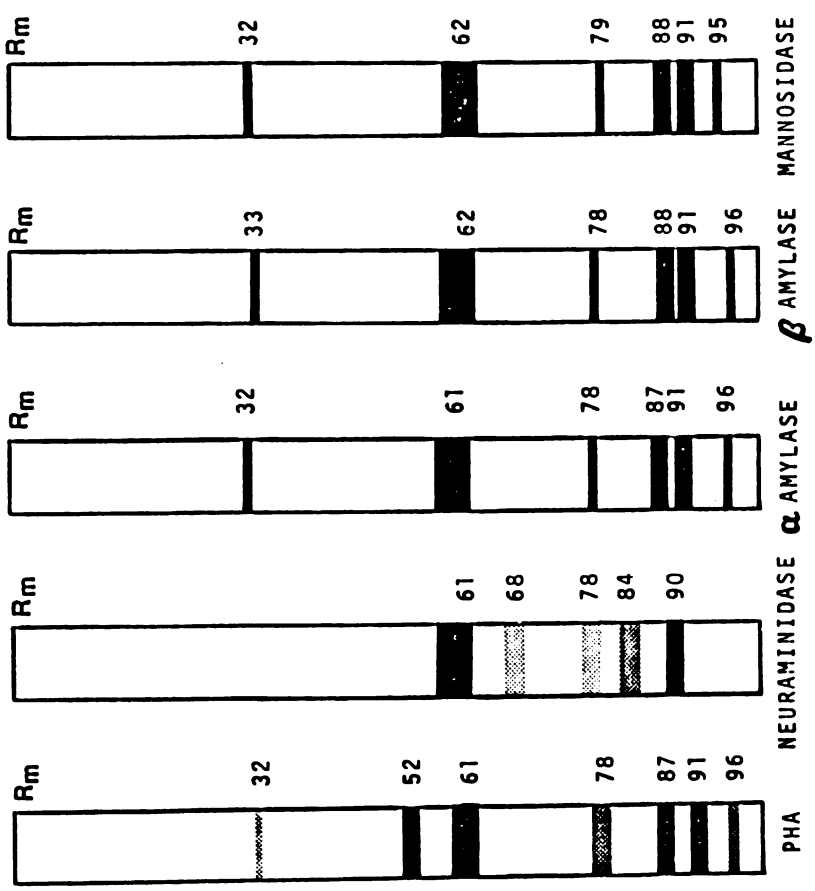
(R_m = relative mobility x 100)

Figure 21. SDS-PAGE analysis of PHA-P exposed to various chemical treatments



(R_m = relative mobility x 100)

Figure 22. SDS-PAGE analysis of PHA-P exposed to various proteolytic enzymes



(R_m = relative mobility x 100)

Figure 23. SDS-PAGE analysis of PHA-P exposed to various enzymatic treatments

gel pattern similar to the untreated protein. In this case, the reduction in molecular weight due to the loss of some mannose is too small to be observed in this study. Neuraminidase treatment also yielded a pattern similar to the PHA-P except that the band near 0.30 was missing. It is interesting to note that R_m of 0.30 corresponds to a molecular weight of approximately 58,000 daltons. It may be possible that the terminal sialic acid residues in the oligosaccharide chain are somehow involved in the intrachain association of the native PHA-P. If they were, it is likely that neuraminidase treatment, which removes terminal sialic acids, would cause the disruption in the PHA-P quaternary structure. In that case, it is also likely that a dimer of PHA subunits which would weigh approximately 58,000 daltons, would be disrupted and no longer observable in the SDS gel.

Conclusions

Purified PHA is stable to freezing and retains full activity for at least seven months when stored at -3°C. However, it is more sensitive to thermal inactivation than the native protein in the whole cotyledons. The thermal inactivation of this lectin is very rapid at temperatures of 80°C or greater and can be described at 70°C by the regression equation :

$$\% \text{ PHA Activity} = 102.18 - 9.87 \text{ (hours at } 70^{\circ}C)$$

The inactivation of PHA at 70°C follows first order kinetics as evidenced by the linearity ($R = -0.999$) of the plot of % PHA activity versus time.

Purified PHA is also strongly affected by chemical treatment.

Treatment for three hours with 2 M sodium chloride or with a pH 3.0 HCl environment causes a slight but significant ($P<0.01$) decrease in hemagglutinating activity. Treatment with 5 % mercaptoethanol causes a 20 % reduction in hemagglutinating activity. The most effective chemical agents for reducing the hemagglutinating activity of PHA-P were urea and a pH 12.0 sodium hydroxide solution, corresponding to 39 and 65 % reduction, respectively after three hours of treatment.

Purified PHA is inactivated by a variety of enzymatic treatments. Treatment with proteolytic enzymes for one to three hours consistently resulted in large decreases (88 - 98 %) in hemagglutinating activity. Treatment with carbohydrate hydrolyzing enzymes is not effective for inactivating the hemagglutinating activity of PHA-P. Treatment with neuraminidase caused a slight but non-significant reduction in the hemagglutinating activity. This indicates that sialic acid residues, although they occupy the terminal positions on the oligosacharide chain of PHA, may not be an important determinant of biological activity. Of the the carbohydrate hydrolyzing enzymes tested, α-mannosidase caused the greatest reduction in the hemagglutinating activity, but this change was only significantly different from the control at ($P<0.05$), not at ($P<0.01$). This indicates that the oligosaccharide chain is important for full activity of the lectin but is not the major determinant of hemagglutinating activity.

Large decreases in the hemagglutinating activity of purified PHA may correspond to a decrease in the enteral toxicity of this lectin. If hemagglutinating activity is correlated to toxicity, it would appear that high pH treatment of beans may be a possible approach for investigating new processing treatments and procedures.

STUDY THREE: EFFECT OF THERMAL EXTRUSION AND ALKALI PROCESSING ON THE HEMAGGLUTINATING ACTIVITY OF DRY BEANS

Introduction

Dry beans (Phaseolus vulgaris) are an important source of protein and other nutrients in many populations. Although they are generally deficient in the sulfur containing amino acids, they supply lysine and therefore have historically been used to complement cereal grains in vegetable-based diets in underdeveloped and emerging nations.

A major drawback to bean consumption is their relatively low digestibility compared to animal proteins (Wolzak et al., 1981a and b, Gomez Brenes et al., 1975). Another drawback is the presence of antinutritional components and enzyme inhibitors in dry beans (Liener, 1978; Liener et al., 1976).

Many processing operations have been investigated for their ability to improve the nutritional quality of P. vulgaris varieties for human consumption. Dry bean digestibility can be substantially improved by heating for 10 to 20 minutes at 121^{o}C (Gomez Brenes et al., 1975). The effect of thermal processing on the antinutritional characteristics has also been investigated. The inactivation of dry bean lectins has been shown to improve the protein quality of dry beans for animals (Jaffe and Hanning, 1965; Jaffe and Vega Lette, 1968; Jayne-Williams and Burgess, 1974). The thermal inactivation of these lectins has been most recently shown by Thompson et al. (1983) and Coffey et al. (1985).

The effect of irradiation on the nutritive quality of beans has been investigated by Reddy et al. (1979) and Mancini Filho et al. (1979). Reddy et al. demonstrated that the nutritive value of all types of dry beans was improved by gamma irradiation. Mancini Filho et al. found that the erythrocyte agglutinating component of bean lectin was inactivated more quickly than the lymphocyte stimulating subunit.

Sprouting the dry seeds has also been reported to greatly improve the digestibiblity of red kidney beans (El-Hag et al., 1978). The authors found that sprouting increased the digestibility coefficient from 29.5 % in the raw to 66.4 % in the cooked kidney bean. Chen et al. (1977) reported that the hemagglutinating activity of a number of pea and bean seeds decreased to generally less than 10 % of the level in the ungerminated dry seed.

The use of alkaline soaking treatment has been used for centuries in the preparation of corn in indigenous Indian diets. This treatment improves the lysine availabilty of the corn and therefore improves the protein quality of the diet. Based on this and on the results reported in study two, alkali treatment of dry beans may be a possible approach for improving the protein quality of beans. However, the treatment would increase the protein quality only by increasing the destruction of PHA in legumes. Little is presently known of the effect of alkali on the attributes of cooked beans.

The per capita consumption of dry beans is decreasing in the United States and is presently approximately 4.1 lbs/person/year. Interest in ethnic cuisine is rapidly increasing and Mexican food is one of the most popular growing ethnic styles. The increased popularity of Mexican cuisine may provide an outlet for expanded use of new bean based

products or ingredients providing opportunities for processing beans in novel ways, such as extrusion. However, little information is available on the effect of extrusion cooking on the antinutrients of dry beans.

In this study, the effect of extrusion and alkaline soaking and cooking conditions on the hemagglutinating activity and texture of dry beans (P. vulgaris) is investigated.

Materials and Methods

Type of Beans

The dry beans (P. vulgaris) used in this study were dark red kidney (Montcalm variety), black turtle soup type (Domino variety) and pinto (Oletha variety).

Processing Operations

Extrusion. Dry beans were prepared for extrusion in either of two ways: 1). by cleaning and segregating the whole seeds; or 2). by grinding the cleaned seeds in a Udy cyclone mill to produce a 50 mesh flour. Whole beans and bean flours were introduced to a Creusot-Loire Model 2000 commercial extruder and processed at barrel pressures ranging from 500 to 1200 psi, barrel temperatures from 124^{o} to 200^{o}C, water feed rate from 9 to 20 mL/minute and final product temperature from 116^{o} to 193^{o}C. The processing parameters for each sample run are presented in Table 6.

High pH Soaking and Cooking. In this study, whole kidney beans (Montcalm variety) were soaked overnight in PBS at pH 7.0 and in physiological saline adjusted to pH 12.0 with sodium hydroxide (Beans:soak media, 1:5, W/V). The soaking media was monitored and

Table 6. Selected Extrusion Process Parameters and Product Characteristics for Extruded Dry Bean Products

Sample Run #	Water Feed mL/min	Barrel Temp °C	Product Pressure psig	Product Temp °C	% PHA Activity
			Kidney Bean Flour		
1	20	124	1200	116	77.70
2	20	146	1000	146	44.13
3	20	150	900	133	26.17
4	20	155	800	146	52.83
5	15	165	950	149	50.07
6	12	175	500	171	44.50
7	12	175	500	182	31.27
			Black Bean Flour		
8	20	100	500	149	45.53
9	12	190	570	177	47.67
10	12	200	900	193	37.37
			Whole Black Beans		
11	20	190	900	171	82.93
12	10	195	1000	184	84.50
			Whole Pinto Beans		
13	10	195	1100	189	24.30
14	20	140	1200	177	25.60
			Wole Kidney Beans		
15	20	150	1000	149	84.97
16	12	185	800	160	87.07
17	9	200	700	171	81.90
18	12	200	1000	182	85.47

adjusted as nescessary with sodium hydroxide solution to maintain pH 12.0. Following the soak, the beans were placed in water baths at 76^{o} and 93^{o}C. At two, four and eight hours intervals, samples were withdrawn, cooled immediately, and a saline extract produced. The extracts were then assayed for hemagglutinating activity as described further in this report. In addition, other samples were withdrawn at the same times and evaluated for texture using the Lee Allo Kramer Shear Press (Food Technology Corp., Rockville, MD) as described further in this report.

Hemagglutinating Activity

The hemagglutinating activities of the products produced in this study were determined by the method of Coffey et al. (1985). These experiments were performed in triplicate and duplicate determinations were made on each sample.

Texture

Kramer Shear Press. (Model TR3 Texturecorder, Food Technology Corp., Rockville, MD). The texture of the cooked beans was determined objectively by shearing a 100 g portion of the cooked sample in an Allo-Kramer shear press equipped with a 3000 pound transducer and a number C-15 standard multi-blade shear compression cell. Shear peak heights indicating maximum force to shear cooked beans were recorded and expressed as Kg/g bean.

Electrophoretic Separations

Discontinuous Polyacrylamide Gel Electrophoresis (DISC-PAGE). DISC-PAGE was used to resolve the component peptide patterns of the treated PHA-P. The method used was that of Davis (1964) but staining was accomplished with 0.04 % Coomassie Brilliant Blue G-250 in 3.5 %

perchloric acid overnight. For all DISC-PAGE evaluations, 11 % acrylamide gel concentration was used for the running gel and the proteins were subjected to 1 mA/tube for 10 minutes followed by 3 mA/tube for the remainder of the separation. The gels were destained and stored in 7 % aqeous acetic acid.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was performed to resolve the component peptides of the treated samples according to their molecular weight. The method used was that of Weber and Osborne (1969). For all SDS-PAGE evaluations, 10 % acrylamide gel concentration was used and the gel tubes were allowed to polymerize for 24 hours before use. The tubes were subjected to 3 mA/tube for 10 minutes followed by 8 mA/tube for the remainder of the separation. After development, the gels were stored in 7 % acetic acid. A series of molecular weight standards were run along with the bean extracts and treated PHA-P. The protein standards were obtained from Sigma Chemical Co. (St. Louis, MO) and their molecular weights in daltons were: Phosphorylase B (94,000); Bovin serum albumin (67,000); Ovalbumin (43,000); Carbonic anhydrase (30,000); Soybean trypsin inhibitor (20,000); and α-Lactalbumin (14,400).

Results and Discussion

Extrusion

Hemagglutinating Activity. The statistical evaluation of the data for extrusion as it affects the hemagglutinating activity is summarized in the analysis of variance (Table 7). Based on this analysis, there is a significant effect ($P>0.01$) of processing conditions on the

Table 7. Analysis of variance of the hemagglutinating activity of various extruded dry bean products as measured by the % PHA activity retention

SOURCE	degrees of freedom	mean squares
PRODUCT	17	1693.63**
ERROR	36	7.54
TOTAL	53	548.36

** Significant at the 1 % level

hemagglutinating activity.

Extrusion of whole beans resulted in slight inactivation of hemagglutinating activity. Figure 24 represents the hemagglutinating activity of extruded whole dry beans. Based on a comparison of these extruded products with a standard PHA, whole kidney and black beans have only 82 to 88 % of the activity of native PHA. This level of activity is approximately the level of hemagglutinating activity in the unheated whole Montcalm and Domino pedigrees (Coffey, 1985). In the unheated raw bean, Montcalm has a hemagglutinating activity of 84 % of PHA and Domino has an activity of 82 %. A comparison of the cell means (Figure 24) shows there is no significant difference ($P>0.01$) between extruded whole kidney beans or whole black beans. These data show that the extrusion processes used were insufficient to cause a significant reduction in hemagglutinating activity of whole kidney and black beans. Pinto beans were incorporated as a low lectin standard and they show an activity of 24 to 26 % of the PHA. This level of % hemagglutinating activity is significantly less than kidney or black bean products at the 0.01 level.

A comparison of the micrographs of these extruded products (see Figure 25) shows granular material incorporated in the extruded pellets of whole beans. The presence of these granules represents uncooked portions of cotyledon.

The hemagglutinating activity of dry bean flours show large changes in the hemagglutinating activity with extrusion. For products one, two and three in Figure 26, there is a strong trend in hemagglutinating activity reduction with increasing temperature, at least over the range from 116 to 133^{o}C. One likely reason that there was such a dramatic effect of temperature in this series is that there were fairly high

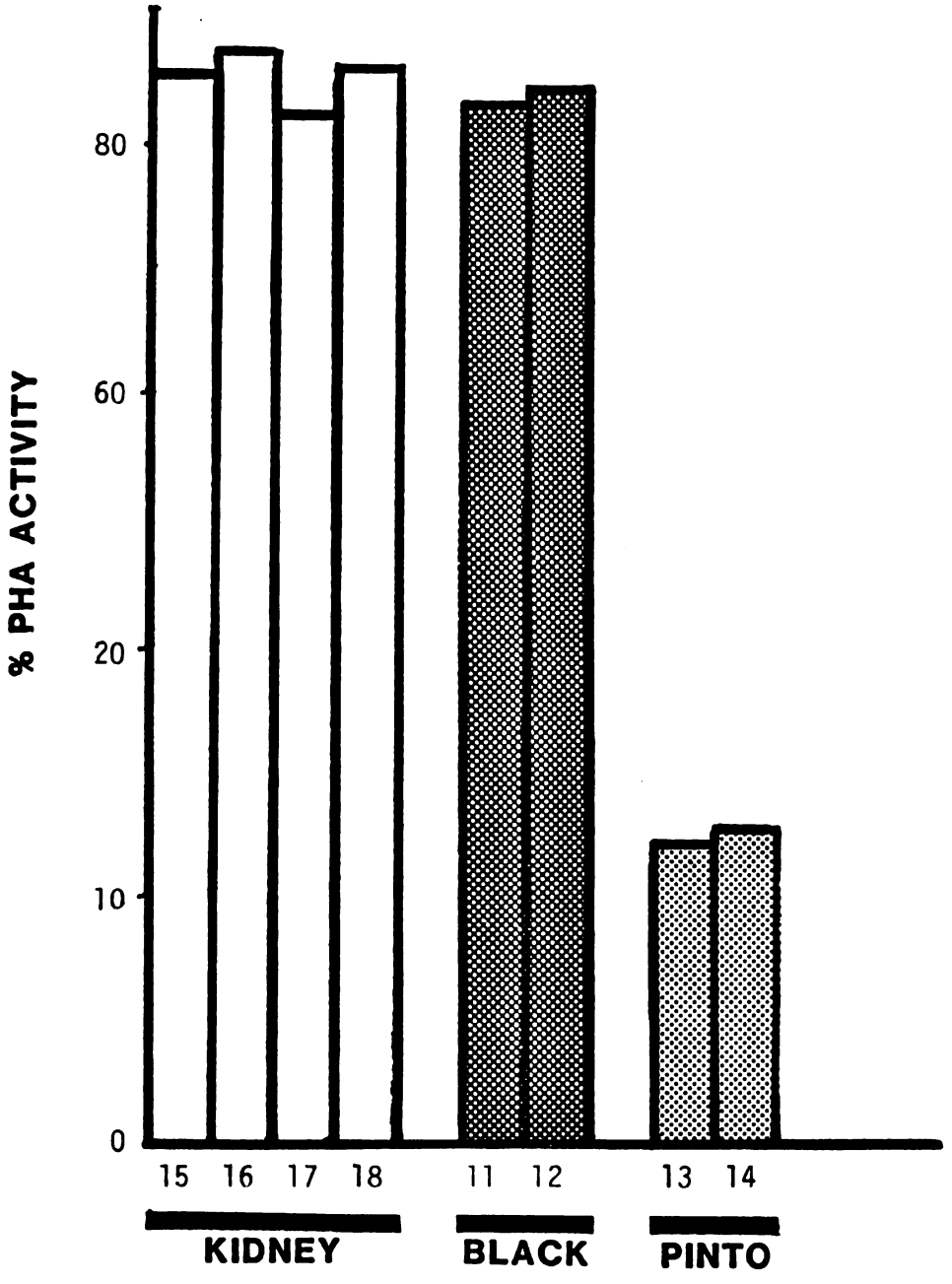


Figure 24. Hemagglutinating activity of extruded whole dry beans

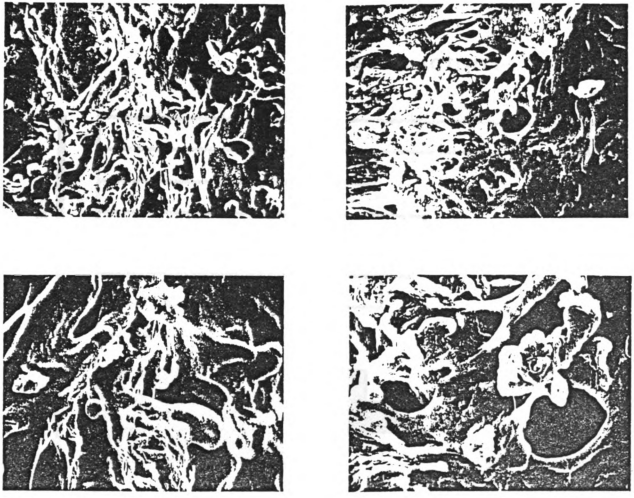


Figure 25. Micrographs of selected extruded dry bean products

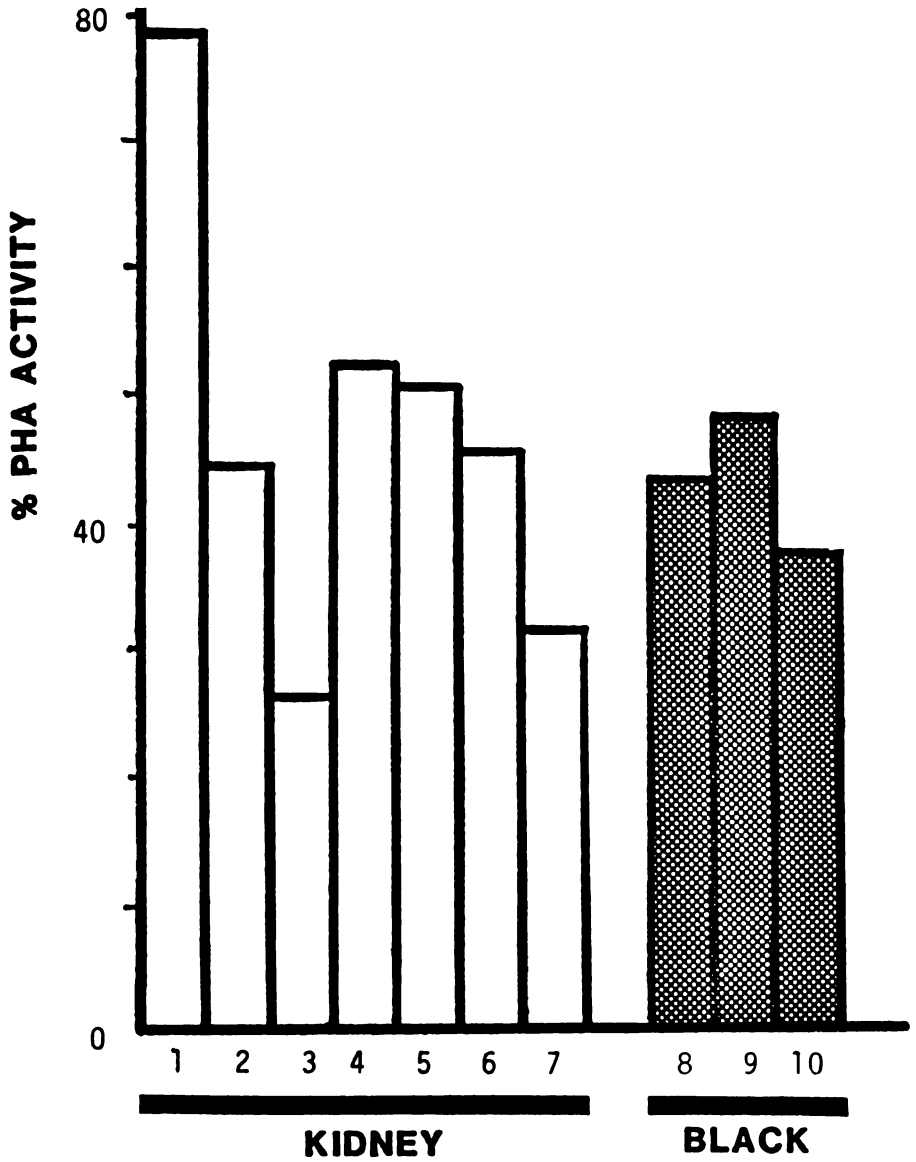


Figure 26. Hemagglutinating activity of extruded bean flours

operating pressures in the barrel.

Likewise, for products five, six and seven there is a strong trend in reduction of the hemagglutinating activity when temperature of the final product increases from 148° to 182°C. No trend was observed with the hemagglutinating activity and barrel pressure in this series. For black bean flour, there seems to be a relationship between the final product temperature, barrel temperature and hemagglutinating activity. Product eight had a value of 43 %, nine had a value of 48 % and 10 had a value of 37 %. This does not correspond to the final product temperature as product eight had a hemagglutinating activity between products nine and 10. However, in the course of the extrusion runs, the barrel temperature dropped to 100°C for product eight whereas it was 190° and 200°C for samples nine and 10 respectively. For the two samples produced at the higher barrel temperature, decreasing hemagglutinating activity corresponded to increasing final product temperatures.

Alkaline Soaking and Cooking

Hemagglutinating Activity. Earlier work has shown a dramatic effect in the hemagglutinating activity when PHA-P was held at pH 12.0 (Coffey, 1985). A similar response is observed in the hemagglutinating activity of whole kidney beans that are soaked and cooked at pH 12.0. As shown in Figure 27, at 76°C, there is a large reduction in the hemagglutinating activity of beans held for eight hours at pH 12.0 versus those at pH 7.0. Cooking for eight hours at pH 12.0 resulted in a retention of only 20 % of the PHA activity versus 97 % for the samples at pH 7.0. There is no significant difference ($P>0.01$) among samples held for two or four hours at either pH level.

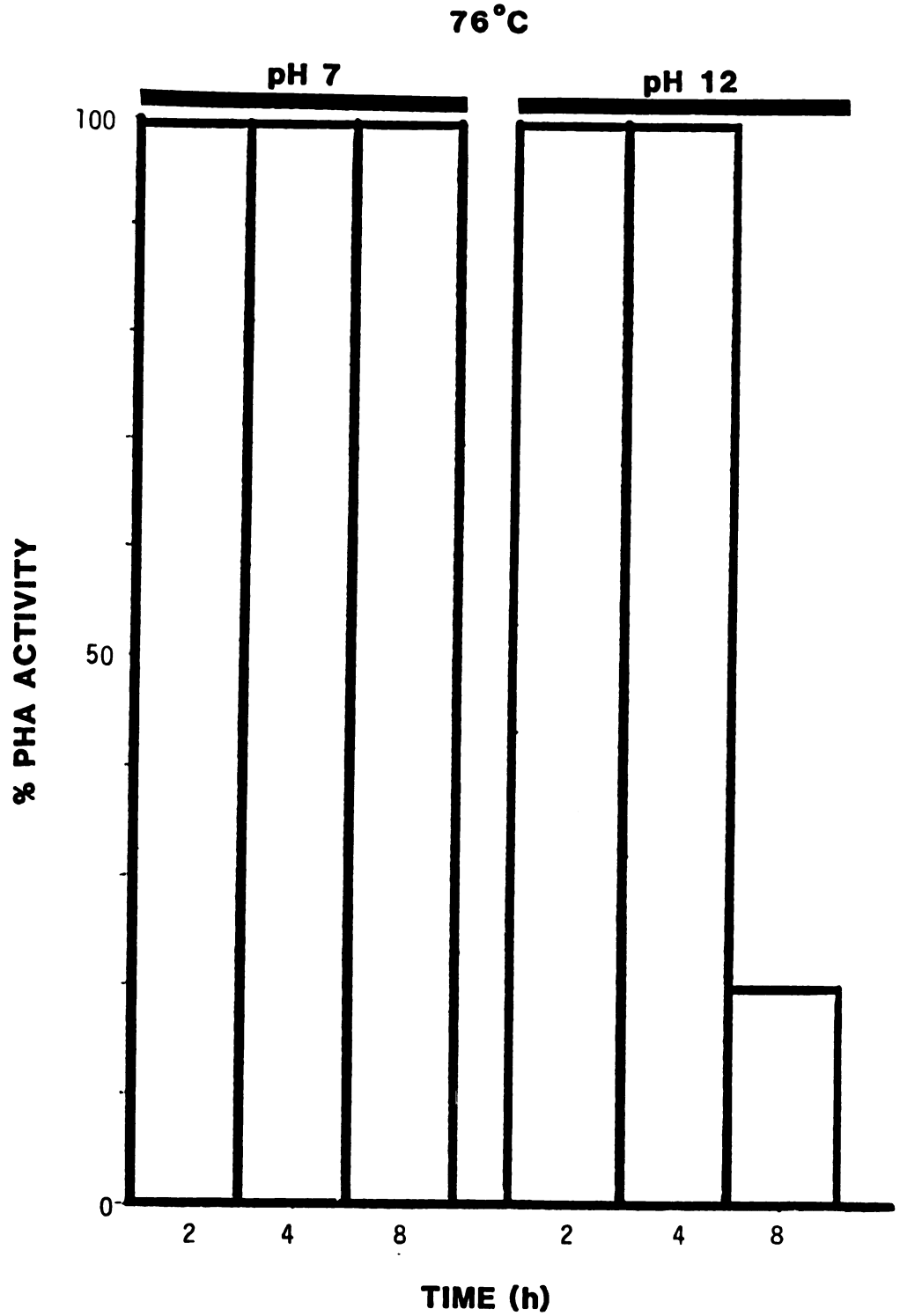


Figure 27. Effect of soak conditions and 76°C heating on the hemagglutinating activity of kidney beans

At 93°C, the situation is similar in that there is greater retention of hemagglutinating activity at pH 7.0 than at pH 12.0 after a two hour cooking time (Figure 28). For samples held two hours at pH 7.0, there was a 53 % retention of hemagglutinating activity. Samples held at pH 12.0 for two hours had no remaining activity. All samples held for four or eight hours at both temperatures had no remaining activity either.

The analysis of variance for the effect of pH and temperature on hemagglutinating activity on kidney beans is summarized in Table 8. There are significant effects ($P>0.01$) due to temperature, pH and time. In addition, there are significant differences ($P>0.05$) for the interactions of temperature x time and pH x time.

Texture. An analysis of the cooked bean texture was performed and the results are summarized in Figure 29. At both temperatures tested, the texture of the high pH beans was softer than those soaked and cooked at neutral pH. For the beans cooked at 76°C, there was a linear correlation of shear force to cooking time for samples at both pH levels. At pH 7.0, the correlation coefficient of the data points and the regression equation describing the line were:

$$r = -0.995$$

$$Y = 1140 - 55.2 \text{ (cooking time in hours)}$$

At pH 12.0 the following values were obtained:

$$r = -0.999$$

$$Y = 883 - 62.9 \text{ (cooking time in hours)}$$

These values indicate that from two to eight hours of cooking time at this temperature, the beans soften more rapidly at pH 12.0 than pH 7.0 and at any particular time the pH 12.0 beans will be softer than

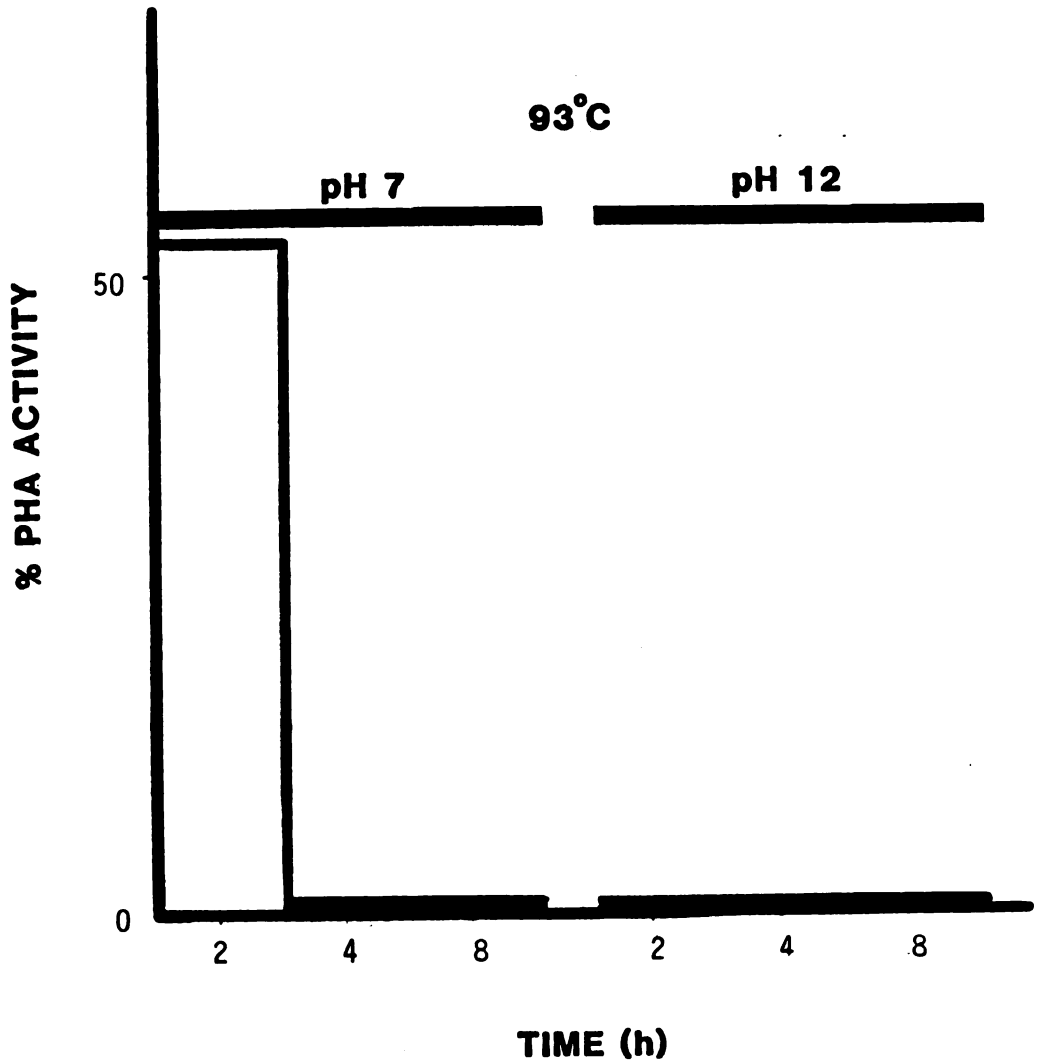


Figure 28. Effect of soak conditions and 93°C heating on the hemagglutinating activity of kidney beans

Table 8. Analyses of variance for heating parameters on the texture and hemagglutinating activty of kidney beans.

ANALYSIS OF VARIANCE OF EFFECT OF HEATING PARAMETERS ON TEXTURE OF KIDNEY BEANS

SOURCE	degrees of freedom	mean squares
TEMPERATURE	1	11,630,000.00**
pH	1	1,343,005.44**
TIME	2	2,371,175.69**
TEMPERATURE X pH	1	86,240.11**
TEMPERATURE X TIME	2	23,590.58**
pH X TIME	2	1096.53
ERROR	26	704.42
TOTAL	35	82,000.44

** Significant at the 0.01 % level

ANALYSIS OF VARIANCE OF HEATING PARAMETERS ON THE HEMAGGLUTINATING ACTIVITY OF KIDNEY BEANS

SOURCE	degrees of freedom	mean squares
TEMPERATURE	1	49,424.70**
pH	1	4323.06**
TIME	2	3111.30**
TEMPERATURE X pH	1	171.17
TEMPERATURE X TIME	2	1156.61*
pH X TIME	2	1189.68*
ERROR	26	241.04
TOTAL	35	2031.46

** Significant at the 1 % level
* Significant at the 5 % level

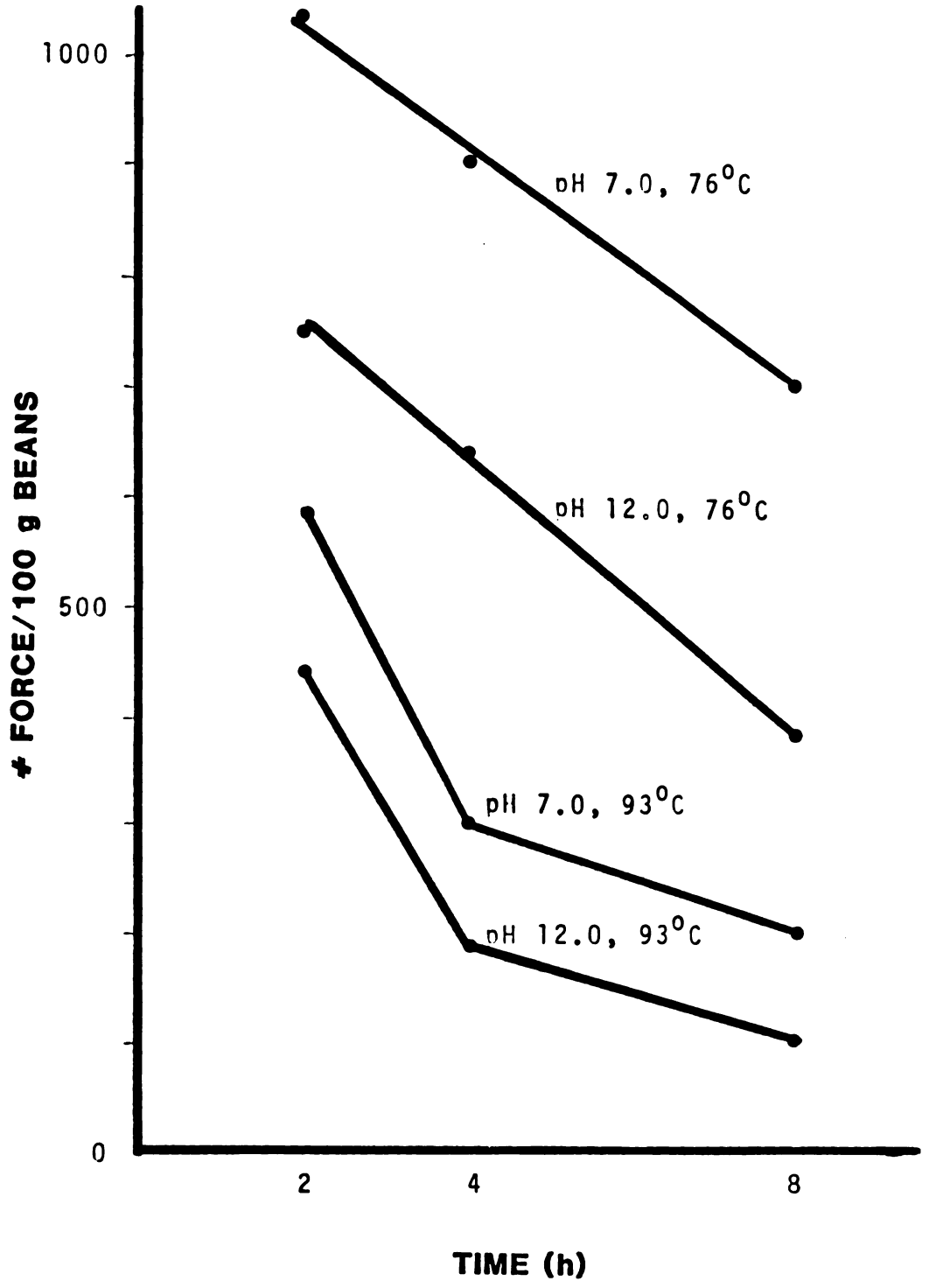


Figure 29. Effect of pH and thermal treatment on the texture of cooked kidney beans

those at pH 7.0.

At a cooking temperature of 93°C, the same relationship was observed. At every time tested, the pH 12.0 beans were softer than those at pH 7.0. At this temperature however, there was no linear or logarithmic correlation of shear force to cooking time. Figure 29 indicates that at eight hours, both samples are approaching a minimum shear value.

Coffey et al. (1985) reported the minimum palatability for cooked kidney beans was 250 lbs/100 g bean. Using this figure and the regression equations for 76°C, beans cooked at pH 12.0 would reach minimum palatability at:

$$\text{Time} = (250 - 883)/-62.9 = 10.06 \text{ hours}$$

For samples cooked at pH 7.0, the time required is:

$$\text{Time} = (250 - 1140)/-55.2 = 16.12 \text{ hours}$$

To summarize the 76°C data, soaking and cooking beans at pH 12.0 speeds the softening of the seeds and decreases the time required to reach minimum palatability by approximately six hours or by 62.4 %.

For 94°C cooking, the minimum palatability can be estimated from Figure 29. At pH 12.0, a shear value of 250 lbs would be expected at approximately 3.5 hours. At pH 7.0, it takes approximately six hours. To summarize the data at 94°C, soaking and cooking beans at pH 12.0 speeds the softening of seeds and decreases the time required to reach minimum palatability by approximately 2.5 hours or by 58.3 %.

The strong relationship between pH and texture and pH and residual hemagglutinating activity suggest that alkali treatment of beans may be a possible approach for processing to improve the nutritional value of dry beans. A comparison of these results with those of the

hemagglutinating activity determinations show that at 76°C (Figure 27), eight hours at pH 12.0 was sufficient to reduce the % PHA activity by approximately 80 % whereas at pH 7.0 for the same time, there was no reduction in activity. For beans cooked at 94°C, this argument becomes relatively unimportant because the hemagglutinating activity is reduced to zero before the beans reach a palatable texture (Figure 28).

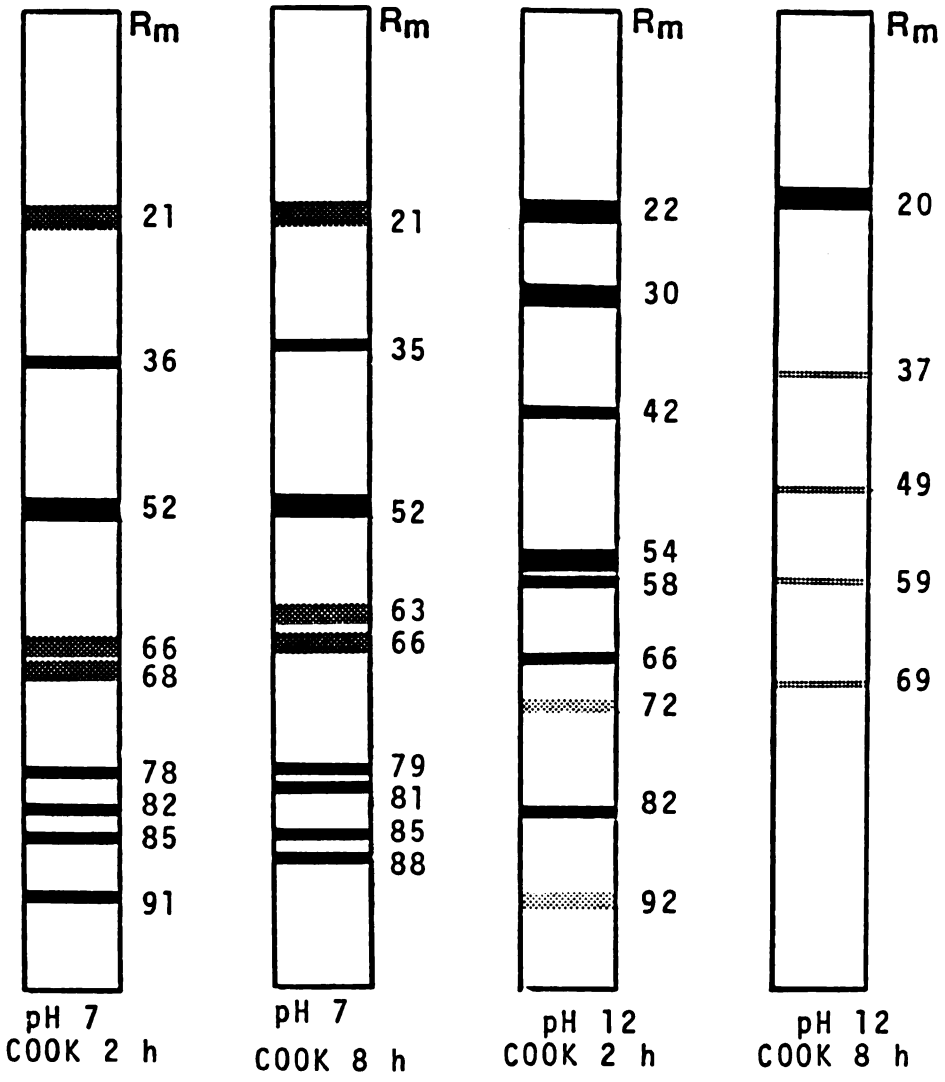
Electrophoretic Analyses

Alkaline Soaking and Cooking. The SDS-PAGE gels of the beans soaked and cooked at pH 12.0 are shown in Figure 30. There are differences for both two and eight hour cooking times for the pH 12.0 beans. For beans held at pH 7.0, the patterns were the same as the control bean extracts and were essentially unchanged during the heating period. For pH 12.0 samples cooked two hours, there were major bands at 0.22, 0.30, 0.42, 0.54, 0.66 and 0.82. For beans held for eight hours at this pH, there was one major band at 0.20 and very faint bands at 0.32, 0.49, 0.59 and 0.69. This indicates that the proteins are being digested and only some of the larger ones (lower R_m values) are remaining.

Extrusion. The results of the SDS-PAGE analysis of the extruded products is illustrated in Figures 31 and 32. According to the patterns produced, there was no easily identified change or correlation with decreasing hemagglutinating activity.

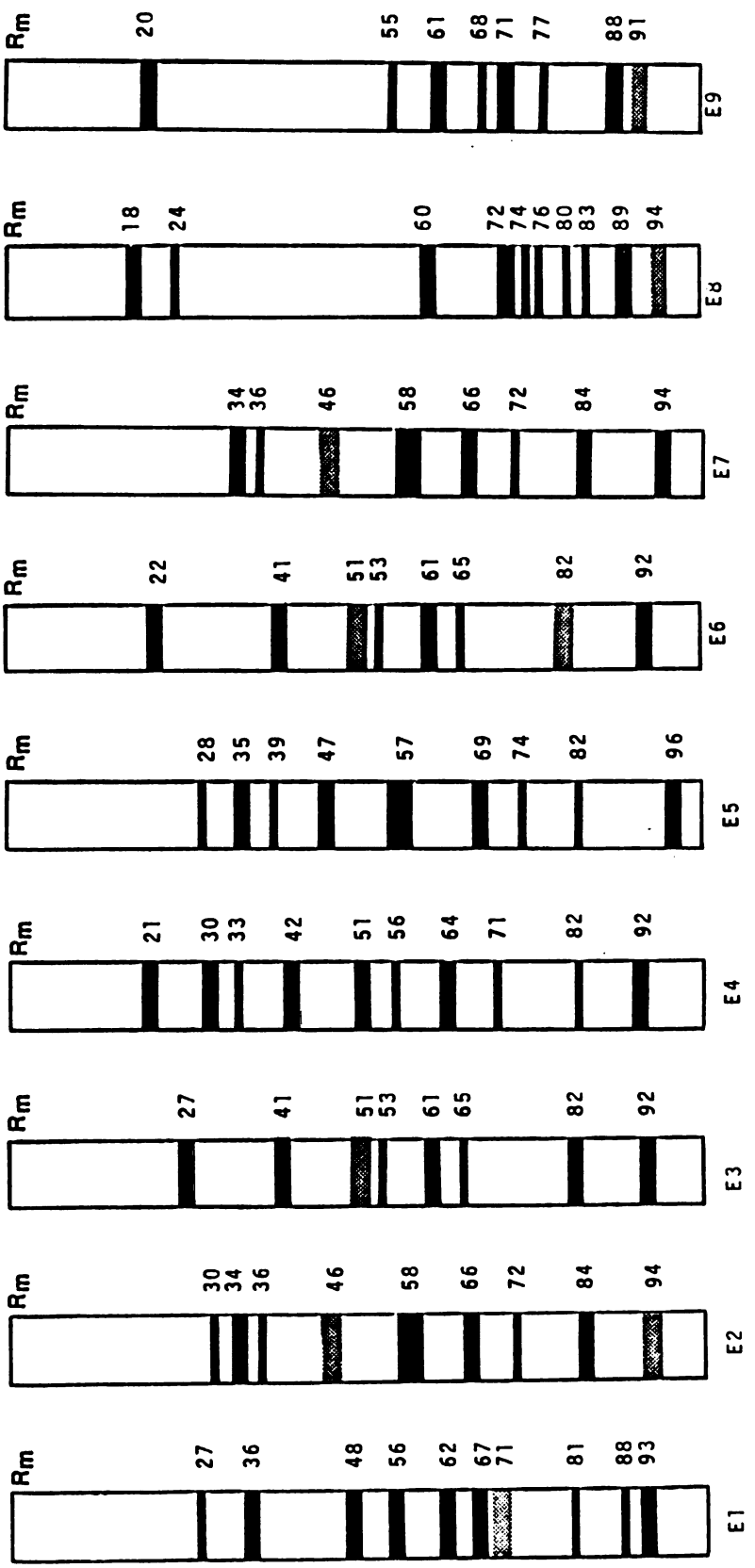
Conclusion

There is considerable variation in the effect of extrusion on dry beans due to changes in the processing parameters. For bean flours, the



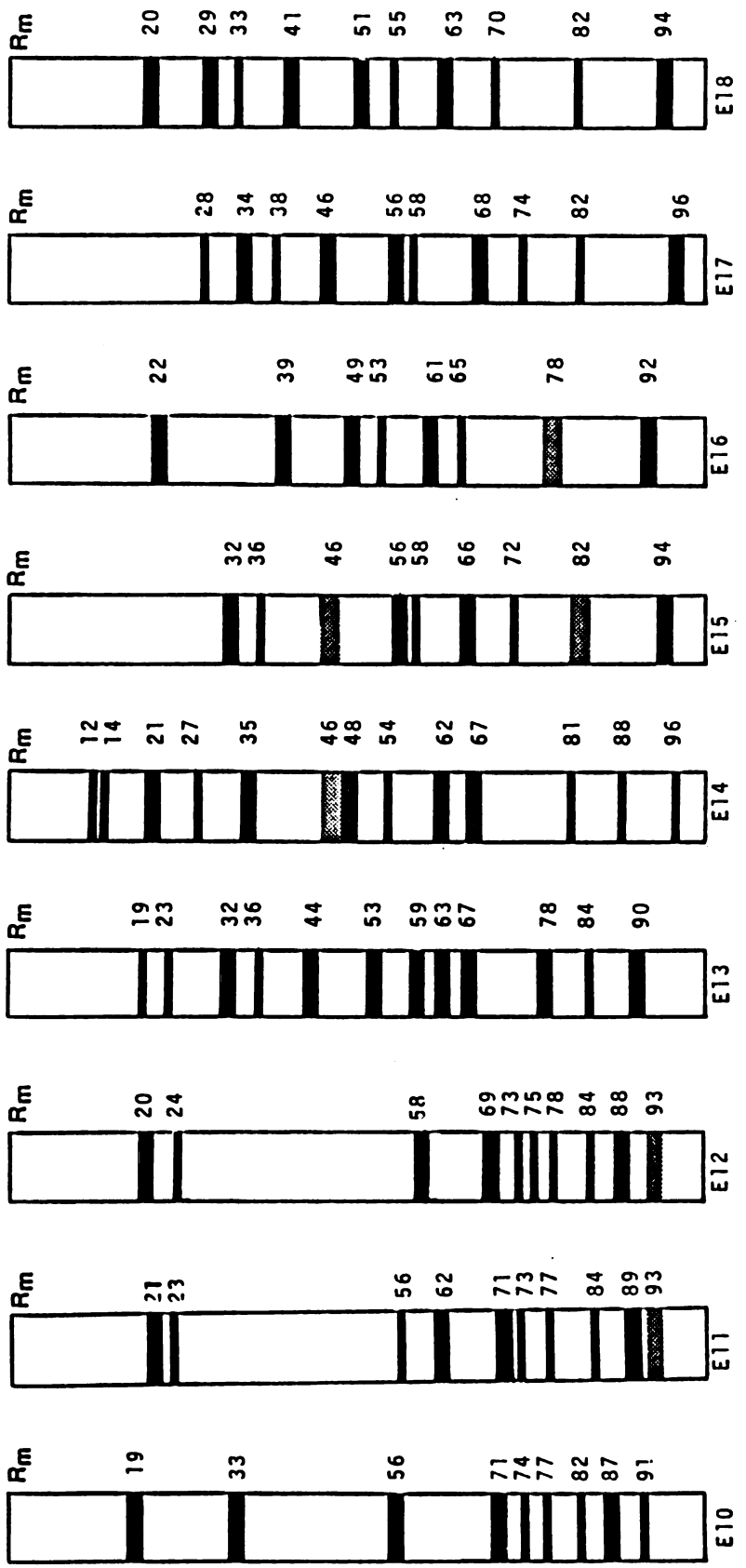
(R_m = relative mobility x 100)

Figure 30. Electrophoretic analysis of kidney beans cooked at pH 7 and pH 12 (SDS-PAGE)



(R_m = relative mobility x 100)

Figure 31. SDS-PAGE analysis of extruded bean products (products 1 - 9)



(R_m = relative mobility x 100)

Figure 32. SDS-PAGE analysis of extruded bean products (products 10 - 18)

hemagglutinating activity decreases with increases in the final extruded product temperature. In addition, internal barrel pressure enhances the effect of final product temperature on the residual hemagglutinating activity. For example, extruding flour at 121°C and 1000 psi results in a % PHA activity retention of 44.13 %. This is not significantly different ($P>0.01$) than a retention of 44.50 % following extrusion at 171°C and 500 psi. The most effective extrusion parameters for bean flours are relatively high pressure (900 - 1200 psi) and temperatures of 270 - 300°C.

Extrusion of whole beans under the conditions employed in this study (150 - 185°C, 700 - 1200 psi) did not result in efficient reduction in hemagglutinating activity. Extruded whole kidney beans and whole black beans showed the presence of uncooked cotyledon particles and retained from 82 - 88 % PHA activity. There was no apparent correlation of hemagglutinating activity with process conditions for whole bean extrusion.

Soaking and cooking kidney beans in alkaline media reduces the hemagglutinating activity more effectively than the same thermal treatment at neutral pH. At 76°C, samples treated at pH 12 retained 20 % PHA activity while samples at pH 7.0 retained 97 %. At 93°C, samples treated at pH 12.0 retained no detectable activity whereas those treated at pH 7.0 retained 53 % of the original activity.

In addition to the increased inactivation of PHA activity, high pH treatment results in significantly softer beans. The regression equation describing the texture of beans cooked at pH 7.0 and 76°C is:

$$Y = 1140 - 55.2 \text{ (hours at } 76^{\circ}C)$$

The regression equation for beans cooked at pH 12.0 and 76°C is:

$$Y = 883 - 62.9 \text{ (hours at } 76^{o}C)$$

The time required to reach an acceptable tenderness based on a minimum palatability end point was reduced when beans were cooked at a pH of 12.0. Beans cooked at pH 12.0 required only 60 % of the time needed for those cooked at pH 7.0 to reach the same textural end point.

These are important considerations for dry bean preparation. A persistent problem in Guatemala and other Central American nations is the development of the hard-to-cook phenomenon in dry beans. This is the condition where dry beans fail to hydrate and soften during cooking. The resultant hard-to-cook beans are difficult to eat and therefore represent a dietary and economic loss for the consumers in those areas (R. Bressani, personal communication). If a simple treatment preventing or ameliorating the hard-to-cook phenomenon could be developed, it would represent a significant improvement in the quality of the diet of those consumers. The results of this study indicate that alkaline soaking and cooking may represent a practical approach to solving this problem.

STUDY FOUR: BREEDING LINE STUDY

Introduction

Dry edible beans are an important food crop, especially in the lesser developed countries of Central America, South America, Africa and Asia. They play an important role as a source of high lysine protein to complement grain based diets. Because of this, bean production is being encouraged in the rural areas of many lesser developed countries. One way to increase production of dry beans is to introduce higher yielding cultivars to small farmers in those countries. However, dry bean yields in the U.S. have remained constant for the last 20 years, suggesting that a "yield plateau" has been reached. If a yield plateau has been reached, cultivar improvement would be an important method for overcoming it (Hosfield and Uebersax, 1980).

Cultivar improvement is necessary if we are to increase the productivity of dry Phaseolus vulgaris species for human food. Many factors must be considered when breeding to improve legumes. Bressani and Elias (1977) reported that the factor of greatest importance for the selection of food crops was crop productivity. They reported this however, as a function of production per unit of arable land, nutritional importance of the crop and the acceptability of the crop to consumers and processors. It was expressed as follows:

Productivity = Yield x Nutritive Value x Technological Value

Obviously, the first and most important emphasis must be given to developing higher yielding varieties. However, these high yielders must

be agriculturally and nutritionally adequate. One important aspect of nutritional adequacy is digestibility and P. vulgaris varieties are known to have digestibilities in the range of 60 to 80 % depending on the cultivar and method of preparation (Wolzak et al., 1981a and b). If a dry bean could be produced that had significantly higher digestibility than is presently available, it would be an important agricultural and nutritional contribution. Another nutritional factor in dry beans that should be considered is the concentration of lectins. These toxic glycoproteins depress the nutritive and protein quality of the beans. Lectins are resistant to heating (Coffey et al., 1985; Gomez Brenes et al., 1975) and if this component could be bred out of the seed, it might represent an improvement in nutritional quality of dry beans.

This study reports the results of an investigation of the digestibility, hemagglutinating activity and selected food quality attributes for 16 pedigrees of P. vulgaris recently produced at the small seeded dry bean nursery at Michigan State University.

Materials and Methods

Source of Beans

The P. vulgaris varieties used in this study were produced at Michigan State University as part of the international dry bean quality nursery. The seed was produced in four row plots 4.9 m long spaced 50.8 cm apart. Standard practices for fertilizer and herbicide application were followed and pods were harvested manually from the middle two rows of individual plots. The pods were threshed by hand and the seeds stored at room temperature. The seed pedigrees produced for this study

were: 800242, Sanilac, 8217-111-24, Nep 2, BTS, MSU-61380, 8217-V111, Jalpatagua-72, San Fernando, Ica-pijao, Jamapa, FF4-13-M-M-M-M, Protop-pi, Carioca, P766, and Mexico 12-1.

Moisture

Approximately 5 g of bean flour were weighed onto previously dried and tared crucibles and dried to a constant weight at 80°C for 24 hours in an air oven. Percent moisture was determined by weight loss on a fresh weight basis (AACC Method 44-15).

% Moisture = (Moisture Loss (g)/Sample Fresh Weight (g)) x 100

Ash

Dried samples obtained from the above moisture determination were placed in a muffle furnace at 525°C for 24 hours. The uniform white ash was cooled in a dessicator and weighed at room temperature. Percent ash was determined on a dry weight basis (AACC Method 08-01).

Percent Ash = (Residue Weight (g)/Sample Dry Weight (g)) x 100

Hunterlab Color Values

The Hunterlab Model D25-2 Color/Difference Meter (Hunter Associates Laboratories, Reston, VA) standardized with a white tile (L = 95.35, a_L = -0.6, b_L = +0.4) was used to evaluate the color of dry bean varieties. A 100 g sample of dry beans was placed in an optically inert glass cup and covered with an inverted white lined can to keep out extraneous light.

Protein

Micro-Kjeldahl. Approximately 30 mg of bean flour were weighed and analyzed by the standard micro-kjeldahl procedure. Percent nitrogen obtained was then multiplied by 6.25 to obtain % protein (AACC Method 46-13).

In-Vitro Digestibility

Sample Preparation. Samples used included a lot of 16 dry bean varieties which were assayed for protein digestibility raw and after cooking. Raw beans were soaked in distilled water (water/bean, 3:1, v/w) for 18 hours at 5°C. Soaked beans were cooked in the autoclave (15 psi, 121°C) for 30 minutes. Cooked beans were dried in a forced draft oven at 60°C for 18 hours, ground in a Udy cyclone mill and analyzed for protein content by the micro-kjeldahl method.

Procedure. The in-vitro digestibility of the samples was assessed by measuring the extent to which the pH of the protein suspension dropped when treated with a multi-enzyme system (all enzymes were obtained from Sigma Chemical Co., St. Louis, MO) including trypsin (porcine pancreatic, Type IX), chymotrypsin (bovine pancreatic, Type II), peptidase (porcine intestinal) and Streptomyces griseus protease (Type XIV) as described by Wolzak et al. (1981a and b). An amount of sample containing 250 mg of crude protein was weighed into a 100 mL beaker and 40 mL of distilled water were added. The protein suspensions were placed in the refrigerator for two hours before analysis. The enzyme solutions were freshly prepared before each series of tests. The multi-enzyme solution contained 22,704 BAEE units of trypsin, 186 units of chymotyrypsin and 0.052 units of peptidase per mL. This solution was adjusted to pH 8.00 with 0.1 N NaOH and/or HCl while stirring in a 37°C water bath. The protease solution contained 7.95 mg/mL. Both were distributed into tubes containing the amount needed for each assay (4 mL) and placed in an ice bath until used.

Each sample was placed in a 37°C water bath and adjusted to pH 8.00 with 0.1 N NaOH and/or HCl, while stirred constantly with a magnetic

stirrer. The multi-enzyme solution was then added. The pH drop was measured with an Orion Research microprocessor ionalyzer/901 (Orion Research, Cambridge, MA). At exactly 10 minutes of digestion, the bacterial protease solution was added and water of a 55°C water bath circulated to the sample. The pH at 15 minutes of total digestion was recorded and the in-vitro digestibility calculated according to the formula proposed by Wolzak et al. (1981b) for P. vulgaris:

In-vitro digestibility = 289.677 - 32.811 (pH 15 min)

Sodium caseinate was used as a reference sample with each series of tests. Duplicate determinations of each sample were performed.

Hemagglutinating Activity

The hemagglutinating activities of the bean varieties studied were determined by the method of Coffey et al. (1985). All experiments were performed in triplicate with duplicate determinations made on each sample.

Electrophoretic Separations

Discontinuous Polyacrylamide Gel Electrophoresis (DISC-PAGE). DISC-PAGE was used to resolve the component peptide patterns of the treated PHA-P. The method used was that of Davis (1964) but staining was accomplished with 0.04 % Coomassie Brilliant Blue G-250 in 3.5 % perchloric acid overnight. For all DISC-PAGE evaluations, 11 % acrylamide gel concentration was used for the running gel and the proteins were subjected to 1 mA/tube for 10 minutes followed by 3 mA/tube for the remainder of the separation. The gels were destained and stored in 7 % aqeous acetic acid.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was performed to resolve the component peptides of the treated samples according to their molecular weight. The method used was that of Weber and Osborne (1969). For all SDS-PAGE evaluations, 10 % acrylamide gel concentration was used and the gel tubes were allowed to polymerize for 24 hours before use. The tubes were subjected to 3 mA/tube for 10 minutes followed by 8 mA/tube for the remainder of the separation. After development, the gels were stored in 7 % acetic acid. A series of molecular weight standards were run along with the bean extracts and treated PHA-P. The protein standards were obtained from Sigma Chemical Co. (St. Louis, MO) and their molecular weights in daltons were: Phosphorylase B (94,000); Bovin serum albumin (67,000); Ovalbumin (43,000); Carbonic anhydrase (30,000); Soybean trypsin inhibitor (20,000); and α-Lactalbumin (14,400).

Results and Discussion

Seed Color

The HunterLab L, a and b color values are summarized in Table 9. Of the 16 varieties tested, four of them (numbers one through four) were white beans. Samples one, two and four looked like typical navy beans however number four was darker than the first two. Sample three was the lightest of the four white beans but had a typical kidney bean shape. Samples five through 12 were all of the black turtle soup commercial class designation and all were much darker (lower L values) and had more red ($+a_L$) and blue ($-b_L$) characteristics than the white navy types. One of the samples of undefined designation, P 766, looked very much like a

Table 9. Seed quality characteristics for selected varieties of P. vulgaris

SEED PEDIGREE	COMM CLASS DESIG	COLOR L	COLOR a_L	COLOR b_L	SEED WT. 100 (g)	SEED MOIS %	SEED PROT %	MEAL MOIS %	MEAL ASH %
800242	WHITE	63.20	7.95	-0.2	17.68	10.1	24.08	8.44	4.13
SANILAC	NAVY	63.65	7.90	0.3	15.35	10.3	20.80	8.51	4.09
8217-111-24	UDF	65.45	7.05	2.15	16.09.	9.9	26.68	8.56	3.98
NEP-2	NAVY	60.25	8.65	1.25	15.14	10.1	21.31	8.58	4.19
BTS	BTS	16.85	46.85	-55.75	18.71	10.7	18.62	9.02	3.85
MSU-61380	BTS	15.55	50.6	-61.2	18.20	10.8	22.15	9.11	4.14
8217-V111	BTS	16.05	48.7	-58.9	20.98	10.5	23.03	9.54	4.01
JALPATAQUA-72	BTS	15.85	49.6	-59.9	21.72	10.5	26.10	9.55	4.29
SAN FERNANDO	BTS	16.25	48.1	-58.0	17.94	10.6	21.06	9.52	4.24
ICA-PIJAO	BTS	15.6	50.3	-60.85	18.10	10.7	19.95	8.69	4.13
JAMAPA	BTS	16.05	48.85	-59.0	21.09	10.9	24.92	8.86	3.95
FF4-13-M-M-M-M	BTS	15.75	49.95	-60.35	19.72	11.0	20.92	9.09	3.75
PROTOP-PI	PINTO	24.55	32.25	-33.05	27.05	10.0	24.87	8.95	3.89
CARIOCA	PINTO	43.6	19.95	-7.6	22.87	7.2	18.90	8.68	3.99
P 766	UDF	21.85	39.85	-37.7	18.81	11.0	21.49	9.12	3.96
MEXICO 12-1	UDF	45.25	20.95	-4.65	20.28	10.3	19.28	8.78	3.67

brown colored black turtle soup bean. Protop-pi and Carioca are both of the pinto designation however they are very different in color. Protop-pi is a dark bean with a minimum of tan mottling on a dark surface. Carioca has some green-brown streaks on a tan surface and is therefore a fairly light bean. Mexico 12-1 is another bean of undefined designation and has a tan coat similar to that of the Carioca base coat.

Seed Weight

All the seeds produced for this study had average seed weights between 15.35 g and 27.05 g per 100 seeds (Table 9). This is expected as they were derived from the small seeded nursery. For comparison, large kidney bean breeding lines have a seed weight of approximately 60 g per 100 seeds (Hosfield and Uebersax, 1980).

Protein

The seeds investigated had protein contents ranging from a low of 18.9 % for the pinto type Carioca to a high of 26.68 % for an undefined white bean 8217-111-24 (Table 9). These data are in agreement with protein values reported for P. vulgaris varieties (Hosfield and Uebersax, 1980; Agbo, 1982).

Hemagglutinating Activity

Of the pedigrees selected, three have fairly high hemagglutinating activity. They are Protop-pi (94 %), 800242 (83 %) and FF4-13-M-M-M-M (80.5 %). The hemagglutinating activity for all lines is shown in Figure 33 and it ranges from a high of 94 % to a low of 3 %. If it becomes important to breed dry beans for low hemagglutinating activity, a number of these beans would serve as good parents as they contain less than 50 % PHA activity. These low lectin varieties are: Sanilac, San Fernando, Mexico 12-1, Black Turtle Soup, Jamapa, Jalpatagua, Ica-Pijao,

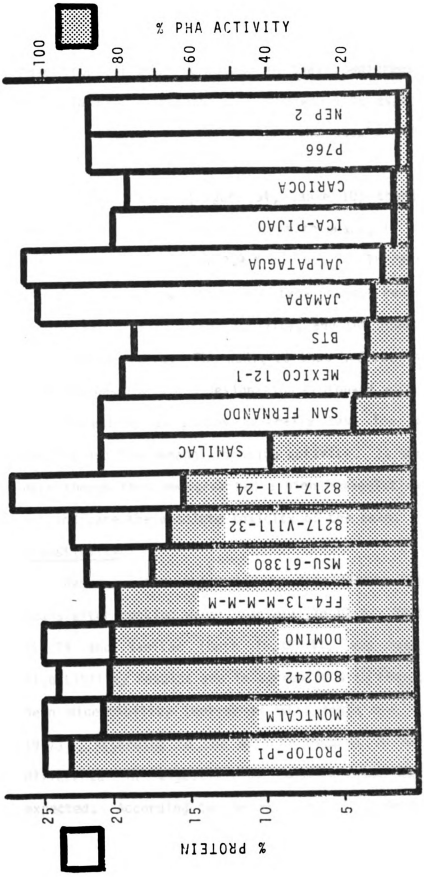


Figure 33. Protein content and hemagglutinating activity of selected P. vulgaris varieties

Carioca, P 766 and Nep-2. It is also fortunate that a number of these have tropical bean germplasm in their pedigrees (San Fernando, Mexico 12-1, Jamapa, Jalpatagua). These would be expected to perform well in tropical climates where many of the people rely on dry beans for a significant part of their diet.

Within classes (Figure 34, Table 10) there is wide variation in hemagglutinating activity. For white beans, it ranges from a high of 84 % for 800242 to a low of 2.9 % for Nep-2. There is a similar wide range of values for black turtle soup type beans. Pedigree FF4-13-M-M-M-M has a high value of 81 % PHA activity, and the lowest value is 5 % for the Ica-Pijao pedigree. The pedigree that had the highest hemagglutinating activity was the pinto designation Protop-Pi with a value of 94 %. This is interesting as pintos generally have low concentrations of toxic lectins and low hemagglutinating activity. It should be noted here that even though this pedigree had a high hemagglutinating activity, it does not indicate the absolute toxicity of the bean.

Digestibilty

Based on the digestibility data (Table 10), the most digestible beans after cooking were: Carioca (78.02 %); Protop-pi (77.95 %); 8217-111-24 and Sanilac (both 76.32 %); and Nep-2 (75.78 %). These digestibility results are in agreement with the published values for dry bean digestibility (Wolzak et al., 1981a and b; Bressani and Elias, 1977). According to the data in Figures 35 and 36, there is a large difference in digestibility between raw and cooked samples, as expected. According to the analyses of variance (Table 11), there are significant effects due to breeding line for digestibility of the raw and cooked bean varieties tested. Also, the digestibilities of white

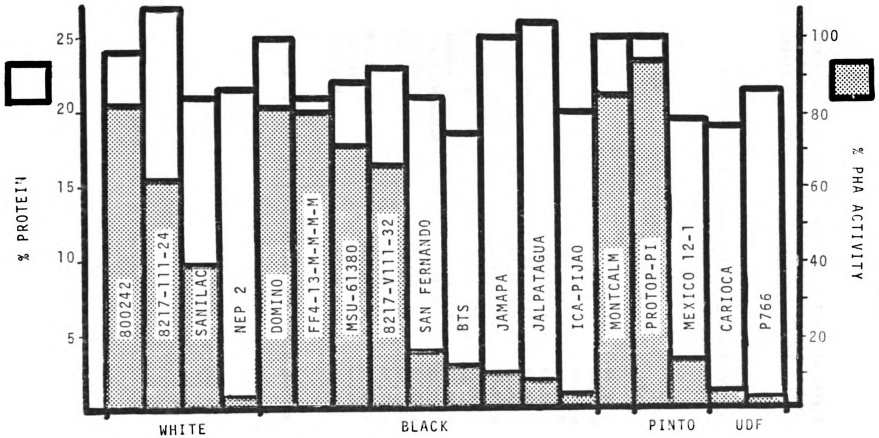


Figure 34. Protein content and hemagglutinating activity of selected P. vulgaris varieties by bean type

Table 10. Statistical summary of the main effects of breeding line on the % PHA activity, raw digestibility and cooked digestibility of selected small seeded P. vulgaris varieties

CELL MEANS FOR THE HEMAGGLUTINATING ACTIVITY AND DIGESTIBILITY OF RAW AND COOKED DRY BEANS

BREEDING LINES	% PHA ACTIVITY	DIGESTIBILITY RAW	DIGESTIBILITY COOKED
800242	17.82 c,d,e	66.58 c	73.60 c,d
SANILAC	43.83	68.55 a,b	75.60 b,c
8217-111-24	65.20 a,b	69.57 a	76.33 a,b
NEP-2	9.25 e	67.05 b,c	75.79 b,c
BLACK TURTLE SOUP	22.47 c,d	60.54	71.11 e
MSU-61380	73.30 a,b	63.53 d	72.69 d,e
8217-V111-32	67.44 a,b	62.82 d	71.43 e
JALPATAGUA-72	17.43 c,d,e	63.87 d	71.77 d,e
SAN FERNANDO	24.61 c,d	65.61 c	71.96 d,e
ICA-PIJAO	15.12 c,d,e	66.62 c	71.23 e
JAMAPA	21.79 c,d,e	63.08 d	73.10 d,e
FF4-13-M-M-M-M	77.16 a	66.72 c	72.18 d,e
PROTOP-PI	94.81	69.24 a	77.94 a
CARIOCA	14.82 c,d,e	68.57 a,b	78.02 a
P766	11.88 d,e	66.71 c	72.13 d,e
MEXICO 12-1	21.19 c,d,e,	65.97 c	72.93 d,e
DOMINO	26.16 c	-----	-----
MONTCALM	61.38 b	-----	-----

Values followed by like letters are not significantly different (P>0.01)

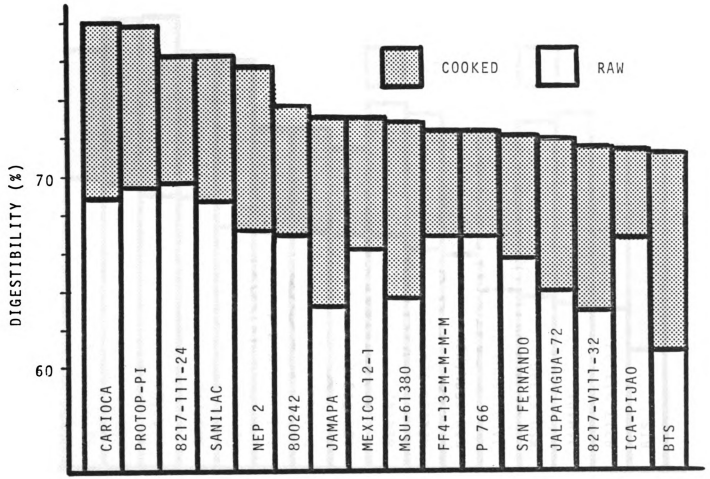


Figure 35. Digestibility of selected P. vulgaris varieties (1)

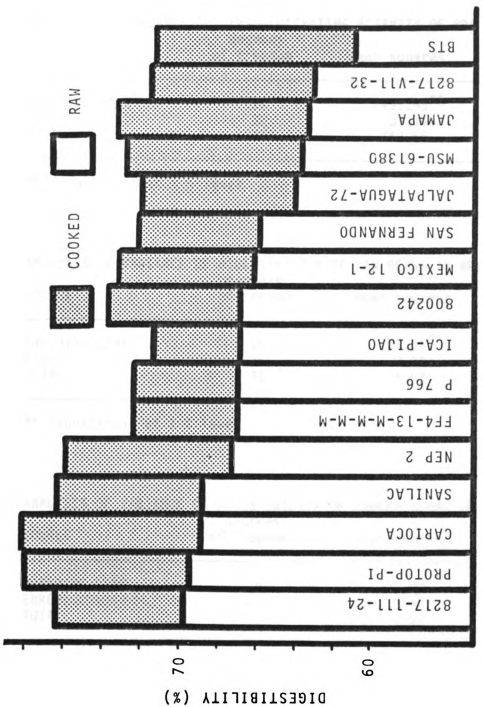


Figure 36. Digestibility of selected P. vulgaris varieties (2)

Table 11. Analyses of variance of hemagglutinating activity and digestibilities of P. vulgaris varieties

ANALYSIS OF VARIANCE OF HEMAGGLUTINATING ACTIVITY OF BREEDING LINES

SOURCE	degrees of freedom	mean squares
BREEDING LINE	17	1493.93**
ERROR	18	33.14
TOTAL	35	742.66

** Significant at 1 % level

ANALYSIS OF VARIANCE OF DIGESTIBILITY OF RAW BEANS IN BREEDING LINES

SOURCE	degrees of freedom	mean squares
BREEDING LINE	15	13.24**
ERROR	16	0.52
TOTAL	31	6.67

** Significant at 1 % level

ANALYSIS OF VARIANCE OF DIGESTIBILITY OF COOKED BEANS IN BREEDING LINES

SOURCE	degrees of freedom	mean squares
BREEDING LINE	15	11.04**
ERROR	16	0.92
TOTAL	31	5.82

** Significant at 1 % level

beans in general are higher than those of colored beans. The lower digestibility of colored beans than white beans has been reported (Aw and Swanson, 1985; Wolzak et al., 1981a and b; Bressani and Elias, 1977) and is presumed to be due to the higher concentration of tannins in the seed coat. The tannins presumably bind to soluble proteins and reduce their digestion. These results support that hypothesis.

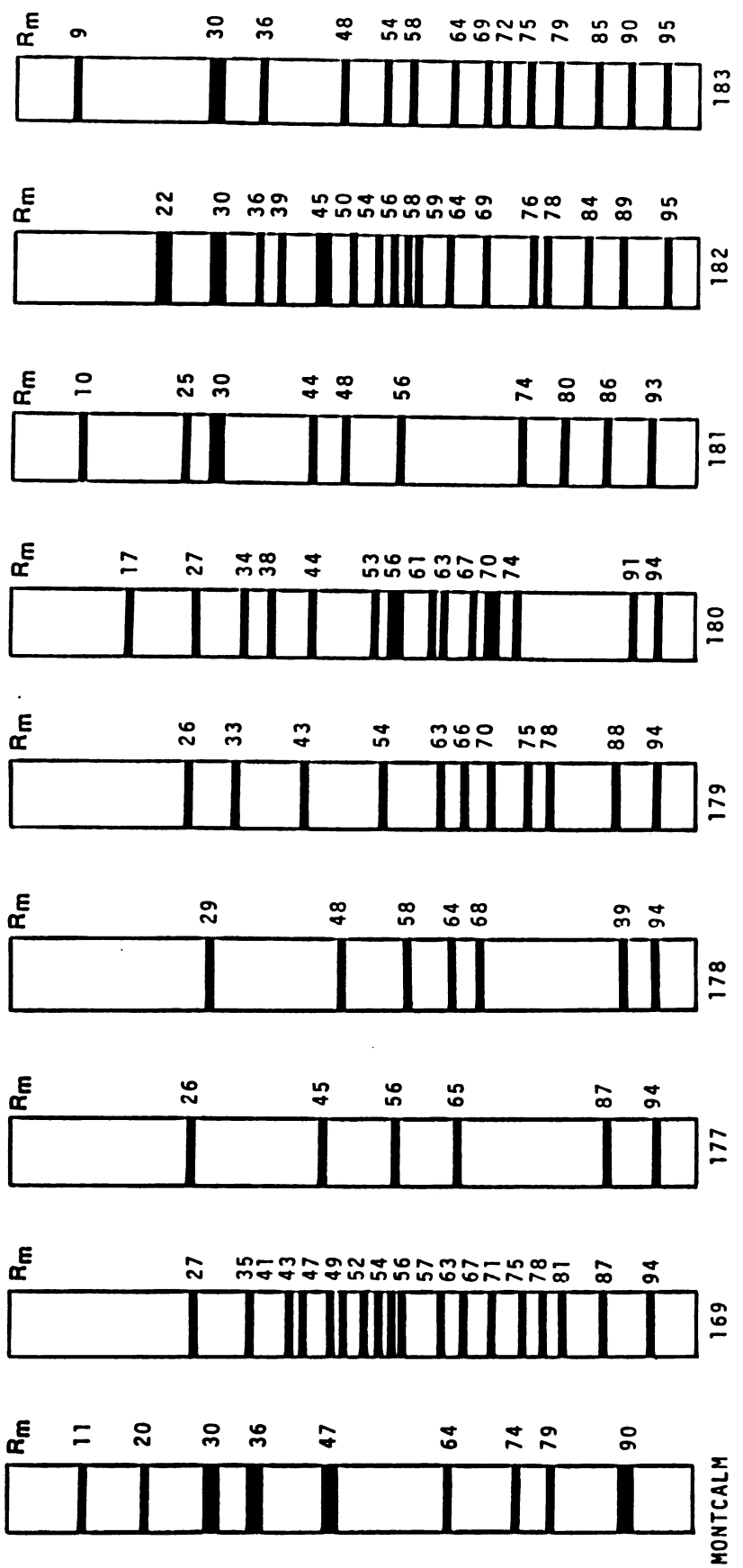
No correlation between digestibility of either raw or cooked beans with protein content or hemagglutinating activity was observed in this sampling of P. vulgaris varieties. However, if lines were to be chosen on the basis of digestibilty for further breeding studies, Sanilac, Nep-2 and 8217-111-24 would be most promising among white navy beans, and Protop-pi and Carioca would be most promising among pintos.

Electrophoretic Evaluation

All lines were subjected to DISC-PAGE and SDS-PAGE and the results are shown in Figures 37 - 40. Based on these results, there doesn't seem to be any correlation with the major bands shown on the gels with either digestibility or hemagglutinating activity.

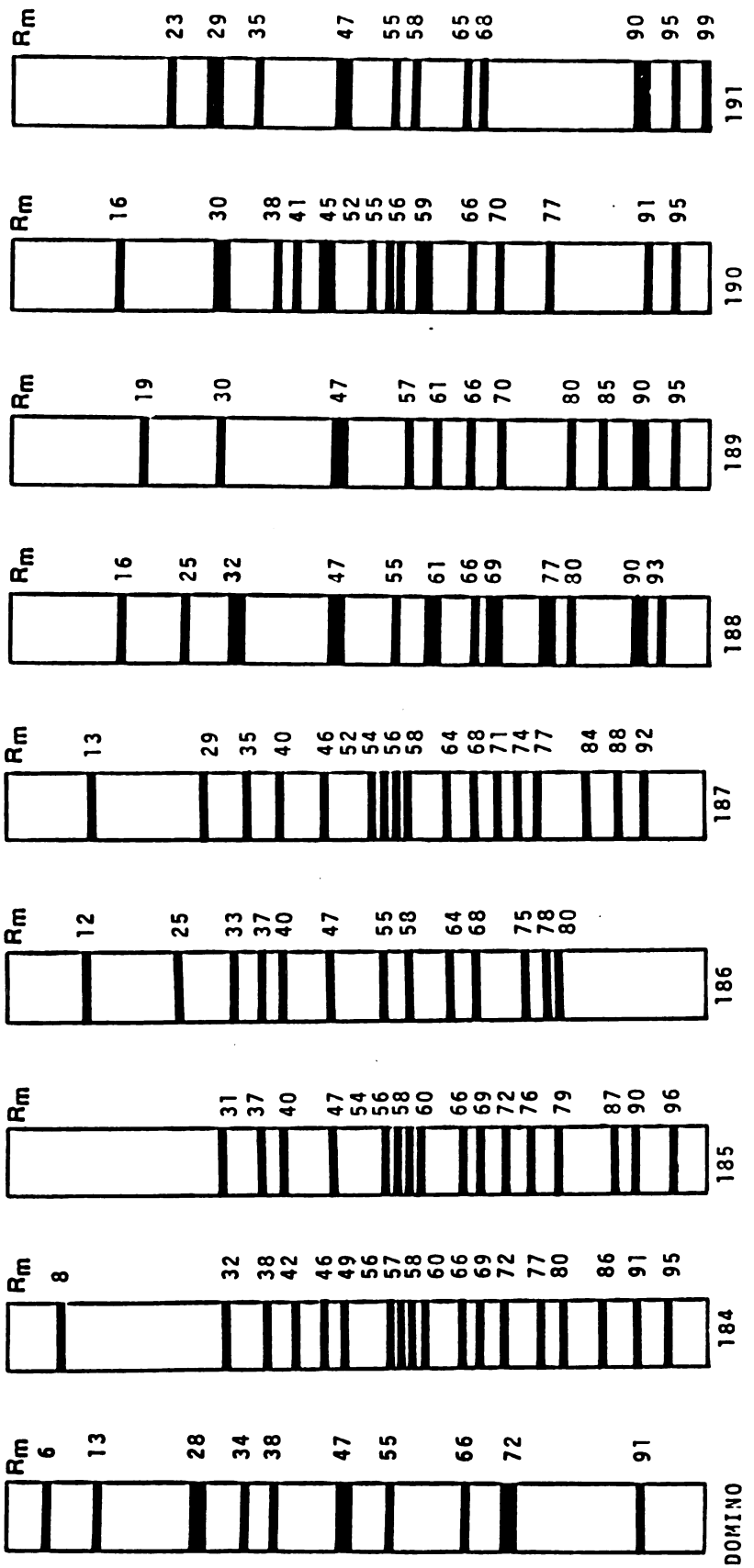
Conclusions

The dry beans produced in this study have acceptable food quality characteristics. The color, protein content and digestibilities of the varieties are appropriate for further consideration in breeding programs. A number of the varieties tested had low hemagglutinating activities and based on this characteristic would be most promising. The low lectin lines were Nep 2, P766, Carioca, Ica-pijao, Jalpatagua, Jamapa, Black Turtle Soup, Mexico 12-1- and San Fernando.



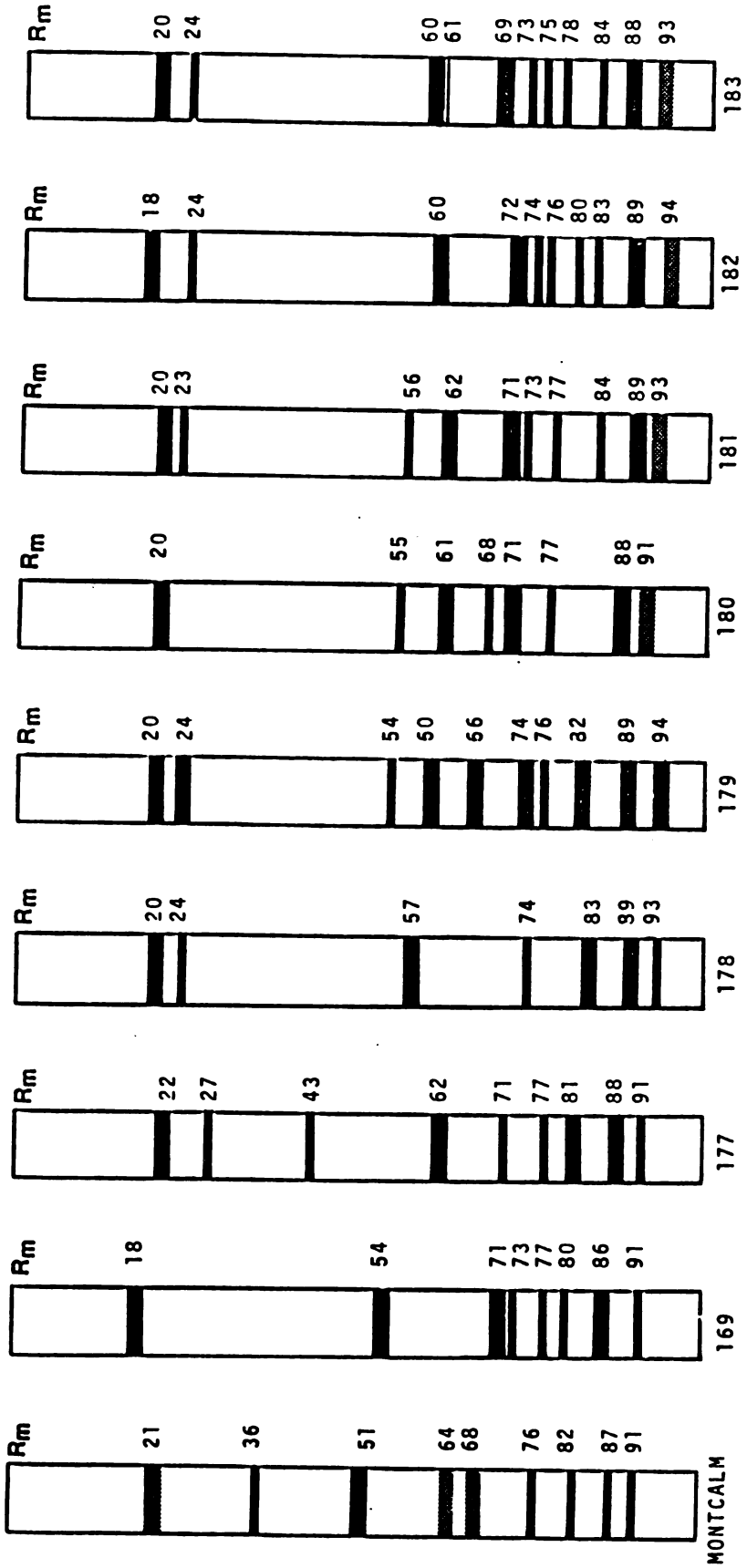
(R_m = relative mobility x 100)

Figure 37. DISC-PAGE analysis of P. vulgaris varieties (1)



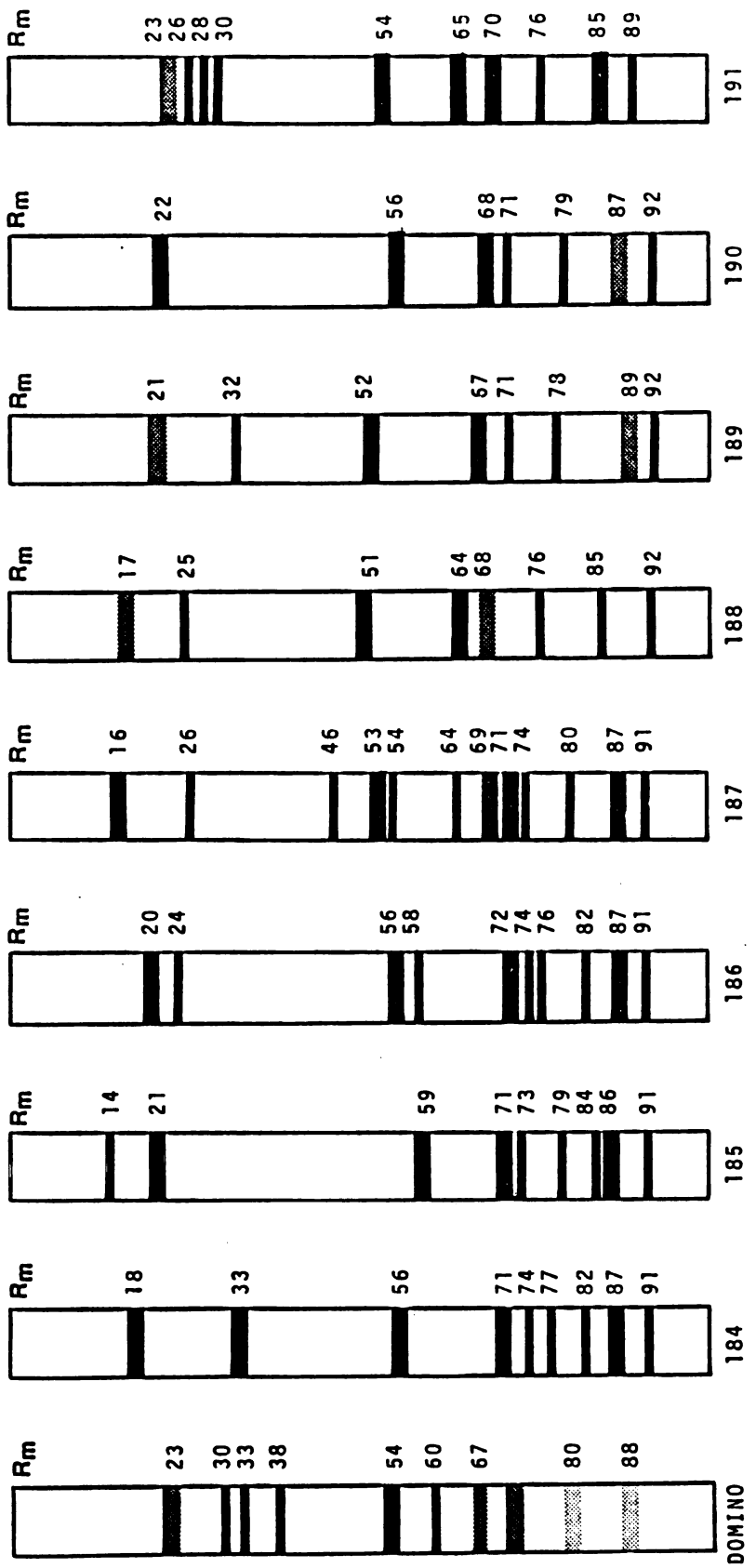
(R_m = relative mobility x 100)

Figure 38. DISC-PAGE analysis of P. vulgaris varieties (2)



(R_m = relative mobility x 100)

Figure 39. SDS-PAGE analysis of P. vulgaris varieties (1)



(R_m = relative mobility x 100)

Figure 40. SDS-PAGE analysis of P. vulgaris varieties (2)

The digestibilties of the varieties varied, with some beans having relatively high values. The most promising candidates for breeding for the digestibility trait would be Carioca, Protop-pi, 8217-111-24, Sanilac and Nep 2.

Electrophoretic analyses of extracts of the beans involved in this study were not conclusive. The patterns produced by DISC-PAGE and SDS-PAGE did not correlate with digestibilty, hemagglutinating activity or protein content. Based on these results, the use of electrophoretic techniques would not be an adequate screening procedure for evaluating newly produced bean varieties for hemagglutinating activity or digestibility.

SUMMARY AND CONCLUSIONS

The electronic cell counter was a sensitive tool for measuring the hemagglutinating activity of kidney beans. The PHA in cooked kidney beans, usually thought to be inactivated by cooking was found in beans exposed to low temperature cooking at times greater than 12 hours. Further research is needed to evaluate any nutritional or physiological impairment resulting from chronic consumption of bean diets containing low levels of active PHA.

Purified PHA was stable to freezing and retained full activity for seven months when stored at -3°C. The thermal inactivation of PHA was rapid at temperatures of 80°C or greater and can be described at 70°C by the regression equation :

$$\% \text{ PHA Activity} = 102.18 - 9.87 \text{ (hours at } 70^{\circ}C)$$

Purified PHA was also strongly affected by chemical treatment. The most effective chemical agents for reducing the hemagglutinating activity of PHA-P were urea and a pH 12.0 sodium hydroxide solution, corresponding to 39 and 65 % reduction, respectively after three hours of treatment.

Purified PHA was inactivated by proteolytic enzymes. Treatment with carbohydrate hydrolyzing enzymes was not effective for inactivating the hemagglutinating activity of PHA-P. Of the the carbohydrate hydrolyzing enzymes tested, α-mannosidase caused only a slight reduction in the hemagglutinating activity. This indicates that the oligosaccharide chain was not the major determinant of hemagglutinating

activity in PHA.

Decreased hemagglutinating activity of purified PHA may correspond to decreased enteral toxicity of this lectin. If hemagglutinating activity is correlated to toxicity, high pH treatment of beans may be a possible approach for investigating new processing treatments and procedures.

There was considerable variation in the effect of extrusion on dry beans due to changes in the processing parameters. The hemagglutinating activity of bean flours decreased with increased product temperature and internal barrel pressure enhanced this effect. The most effective extrusion parameters for bean flours were relatively high pressure (900 - 1200 psi) and temperatures of 270 - 300^{o}C. Extrusion of whole beans under the conditions employed in this study (150 - 185^{o}C, 700 - 1200 psi) did not result in efficient reduction in hemagglutinating activity. There was no apparent correlation of hemagglutinating activity with process conditions for whole bean extrusion.

Soaking and cooking kidney beans in alkaline media reduces the hemagglutinating activity much more effectively than the same thermal treatment at neutral pH. In addition to the increased inactivation of PHA activity, high pH treatment results in significantly softer beans after cooking. The regression equation describing the texture of beans cooked at pH7.0 and 76^{o}C is:

$$Y = 1140 - 55.2 \text{ (hours at } 76^{o}C)$$

The regression equation for beans cooked at pH 12.0 and 76^{o}C is:

$$Y = 883 - 62.9 \text{ (hours at } 76^{o}C)$$

Beans cooked at pH 12.0 required only 60 % of the time needed for those cooked at pH 7.0 to reach the same textural end point.

The dry beans investigated in Study Four had acceptable food quality characteristics. The color, protein content and digestibilities of the varieties were appropriate for further consideration in breeding programs. The most promising low lectin lines were Nep 2, P766, Carioca, Ica-pijao, Jalpatagua, Jamapa, Black Turtle Soup, Mexico 12-1- and San Fernando. The most promising candidates for breeding for the digestibility trait would be Carioca, Protop-pi, 8217-111-24, Sanilac and Nep 2.

Electrophoretic analyses of extracts of the beans involved in this study did not correlate with digestibilty, hemagglutinating activity or protein content. The use of electrophoretic techniques would not be an adequate screening procedure for evaluating newly produced bean varieties for hemagglutinating activity, digestibility or protein content.

RECOMMENDATIONS FOR FURTHER RESEARCH

There are a number of further studies that could be pursued that relate to bean proteins and the improvement in nutritional quality of legumes:

1). It is suggested that a feeding trial be undertaken to determine the effect of denatured PHA on laboratory animals. The PHA could be purified, denatured and then added to a balanced diet or beans could be partially heated and a diet containing a serial addition of the heated beans produced.

2). Further studies concerning the role of high pH treatment on the nutritive quality of dry beans should be pursued. Specifically, the role alkali treatment may have on the inactivation of PHA and the possible role of this type of process for bean preparation in rural Central American populations.

3). The possible role of high pH treatment for the prevention of the hard-to-cook phenomenon is another area to be studied. Consumer acceptance of this type of preparation should also be investigated.

4). The role of thermal extrusion as a dry bean processing operation should be investigated, especially for its potential ability to produce bean products with reduced lectin levels.

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