1993

Isolation and identification of root-inhibiting compounds from corn gluten meal

Dianna Lan-Ying Liu
Iowa State University

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Isolation and identification of robot-inhibiting compounds from corn gluten meal

Liu, Dianna Lan-Ying, Ph.D.
Iowa State University, 1993
Isolation and identification of root-inhibiting compounds
from corn gluten meal

by

Dianna Lan-Ying Liu

A Dissertation Submitted to
the Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Department:  Horticulture
Major:  Horticulture

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1993

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DEDICATION

To my Savior and my Lord Jesus Christ, who is and who was
and who is to come, the Almighty

O Lord, my God
When I consider the work of
Your fingers, what am I, that You are mindful of
And keep as the apple of Your eye.
Your ways are higher than my ways, and
Your thoughts than my thoughts.
Although bread of privation and water of oppression
Were given to me, You never hided Yourself,
And my eyes always beheld You.
Said the Lord, my Creator, to me,
“Do not fear, for I have redeemed you.
I have called you by name; you are mine.”
When I passed through the waters, they did not overflow me.
When I walked through the fire, the flame did not burn me,
For You were with me.
You rescued and brought me out into a spacious place.
You watched over me passing through the valley of weeping
And made it a blessing.
You have crowned the years with Your grace,
And Your paths drip with mercy.

O Lord, my God
You protect the aliens and the oppressed.
You are a refuge for the fatherless and the widow.
I will give my thanks to my Lord because of
Your loving kindness and mercy.
I will praise You who counsels me.
I will dwell in the house of my Lord forever.
May the words of my mouth and
The meditation of my heart please You.
Blessed is the man whose delight is in Your law,
And on Your law he meditates day and night.
Blessed is the nation whose God is the Lord.
May everything that has breath praise the Lord Almighty.
May the whole earth be filled with
Your Glory forever and ever.
Amen and Amen.
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INTRODUCTION

Although chemicals with increased efficacy and safety have been developed in recent years, the agrochemical industry faces continuing and growing criticism over the toxicity and residue problems associated with the use of pesticides (Huppatz, 1990). The use of synthetic pesticides for weed control has recently become a serious environmental concern. This is particularly a problem when these synthetic pesticides come in contact with the public as is the case in turfgrass areas and in the production of food crops consumed by humans.

The judicious application of herbicides has become an integral part of agriculture (Pimentel, 1986a). Public awareness and concern for environmental protection and human safety has led to the search for natural pesticides that provide a greater margin of safety for the public. This includes the search for naturally occurring compounds that are able to inhibit the growth or development of weeds. These materials are potentially non-toxic, natural herbicides that can provide a substitute for, or supplement to, synthetic herbicides.

Allelopathy is a term describing the chemical interaction between plants (Rice, 1984). Its definition has broadened markedly in the past decade and the term may be used to describe tools for biological weed control (Lovett, 1991). Recognition of the economic imperative in developing alternative approaches to agriculture may prove to be one of the most significant and encouraging trends that have emerged during the 1980s. The use of natural pesticides may help to
change the traditional approaches to agricultural science and open the way to the development of sustainable systems (Lovett, 1991).

It has been reported that corn gluten meal, which is a by-product of corn from the wet milling process, is useful as a natural preemergence herbicide and fertilizer material for various plant production systems (Christians, 1993). The primary use of corn gluten meal has been as a feed substance. It would be environmentally and economically desirable to exploit this material as an alternative to conventional herbicides. However, corn gluten meal is quite insoluble in water and this characteristic renders it hard to apply as a herbicide. If the water soluble inhibitory compounds in corn gluten meal can be isolated and identified, they may be useful as preemergence weed controls. They could be used as a substitute for synthetic chemical herbicides or as a supplement to synthetic herbicides to reduce the concentration of these pesticides in the environment.

The overall objective of this research was to search for a herbicidally active and water soluble sample derived from corn gluten meal from which to isolate and identify the bioactive inhibitory compound(s). This dissertation describes the approaches of the objective in 4 phases of study. In the first phase, the phytotoxicity of various samples derived from corn gluten meal and other related crop materials were evaluated using different grass species under controlled environments in order to select the most potent and water soluble sample from which to isolate the herbicidally active component(s). Based on the results from 4 studies, both in greenhouse and growth chamber, the corn gluten meal hydrolysate prepared with bacterial proteinase contained the most potent inhibitory activity for 3 test grass species. The hydrolysate was chosen
for isolation and identification of active compound(s). The objective of the second phase was to develop a purification protocol to extract and isolate the root-inhibiting compounds from enzymatically hydrolyzed corn gluten meal. The isolated bioactive fraction was chemically identified as 5 dipeptides. The third phase included quantitative purification following an established protocol using a perennial ryegrass bioassay that was developed in this research as an activity index. The objective of the final phase was to evaluate the root-inhibiting activity of the 5 identified dipeptides using petri dish and soil bioassays with perennial ryegrass and creeping bentgrass, respectively. Other commercially available dipeptides and constituent amino acids were also evaluated for their root-inhibiting activity under growth chamber conditions.

Two U.S. patent applications based on this work, ISURF #101631 U.S. patent application for *Preemergence weed control using plant gluten hydrolysates* and ISURF #101631 U.S. patent application for *Preemergence weed control using peptides from corn gluten hydrolysate*, have been filed with the Patent Office.
LITERATURE REVIEW

Weed Control

Weeds are any plants growing where they are not wanted. In turfgrass, the term refers to any undesirable plant with a disruptive effect on the aesthetic appearance, or overall utility of a turf. Weed species are classed as annuals, biennials, or perennials. For control purposes, weeds may be divided into the following functional categories; annual grasses, perennial grasses, annual broadleaf weeds, and perennial broadleaf weeds (Turgen, 1985). Weed control is any practice designed either to prevent weed emergence, to reduce weed infestations, or to effect a shift away from undesirable vegetation and toward the desirable plants. Weed control is a matter of degree, ranging from poor to excellent. The degree of weed control obtained is dependent on the characteristics of the weed(s) involved and the effectiveness of the control method(s) used (Anderson, 1983). Eradication, which means a given weed species has been killed or completely removed from a given area and will not reappear unless reintroduced to the area, is ideal but is seldom achieved.

Herbicides

Certain chemicals are capable of killing plants or greatly retarding plant growth. These phytotoxic chemicals are known as herbicides. Herbicides vary in chemical structure, however, nearly all modern herbicides discovered since 1944 are organic chemicals (Anderson, 1983). Organic herbicides will break down in the soil or in the plant within several weeks or months (Turgen, 1985).
Herbicides are commonly grouped on the basis of their characteristic herbicidal activity, application, or chemical similarity. In general, herbicides are either selective or nonselective with respect to the kinds of plants that they affect. Non-selective herbicides will kill all plant life on the plot of soil on which the herbicides are applied. Selective herbicides only kill certain type of plant life, for example weeds, and leave the desirable plant relatively undamaged. One way to selectively eliminate unwanted plants without injuring surrounding plants is to inhibit the germination of the seeds of the unwanted plants in mature stands of a desirable plants.

The method of herbicide use can vary. Preemergence herbicides are applied prior to the unwanted plants emergence from the soil but after the emergence of the desirable plants. The precise time of application will vary, depending upon the area of the country in which the herbicides are applied and the weed species involved (Turgan, 1985). Postemergence herbicides are applied after emergence of the weed. In turfgrasses, many annual grasses are controlled with preemergence herbicides.

**Adverse effects of the use of herbicides**

Herbicides and other pesticides including insecticides, fungicides, and nematicides are used for pest control to reduce the adverse effects of pests. The use of herbicides for weed control has been practiced to some extent since 1927. In the last 40 years, synthetic organic pesticides have become a major component of agricultural, forestry, and turfgrass management systems (Balogh and Anderson, 1992). The synthetic pesticides have been highly effective against pests, while causing little or no damage to the crop. However,
as the use of synthetic pesticides grew, their effectiveness unexpectedly declined (Pimentel, 1986b). Balogh and Anderson (1992) report that environmental problems and human health problems associated with pesticides can be documented. Therefore, serious questions have been raised concerning the real benefit of pest control strategies based solely on synthetic chemicals. The synthetic pesticides, although useful, may be potentially hazardous to crops because of their phytotoxicity, and indirectly to cattle and humans that feed on them (Rizvi et al., 1980). Since pesticides are toxic or biologically active by design, there is a concern regarding their effects on human health and environmental quality (Balogh and Anderson, 1992).

Pesticide residues have been associated with adverse environmental and human health effects. Pesticides can influence the essential functions performed by the natural biota that maintain environmental quality (Pimentel and Edwards, 1986). A growing evidence indicates that small quantities of pesticide residues can migrate from agricultural and turfgrass areas to impact adjacent land, the atmosphere, and water. Pesticides have been found at low levels in surface water and groundwater used for drinking. The EPA estimates that 10.4% of community water systems and 4.2% of rural domestic wells are contaminated with at least 1 of the 126 pesticides included in the analysis (Balogh and Anderson, 1992). It is also found that herbicidal action on aquatic plants may cause excessive algae growth with the release of nutrients from decaying vegetation.

Human pesticide poisonings are clearly the highest price paid for intensive pesticide use. An estimation of 45,000 humans are poisoned by pesticides each year in the United States. Of these, 2,831 are poisoned
seriously enough to be hospitalized (EPA, 1976). This may be expected from the dispersal of pesticides over broad areas of land. Valuable domestic animals are poisoned and various livestock products are also contaminated with the chemicals. Furthermore, an indirect cost of employing pesticides is the reduction of beneficial natural enemies of pests. (Pimentel, 1986a).

**Integrated Pest Management**

Because of the diverse problems associated with 'pesticide-only' control, a return to a more broadly based pest control program has been strongly suggested by many agriculturalists. This philosophy of pest control is called integrated pest management (IPM). It is a pest control method that includes judicious use of pesticide and nonchemical technologies based on sound ecological principles (Pimentel, 1986b). Since Pickett et al. (1958) suggested the need for an ecological approach to pest control, IPM has become the stated policy of the U.S. Department of Agriculture, U.S. Environmental Protection Agency, and the Council on Environmental Quality as well as in the nations of Europe and elsewhere in the world (Pimentel, 1986b). These IPM programs offer a numerous opportunities for improving pest control to benefit farmers, society, and the environment.

Weed control programs that employ the combinations of available weed-control techniques are called integrated weed control programs (Anderson, 1983). They involve the use of two or more weed-control techniques selected from the five general categories: preventive, biological, cultural, mechanical (physical), and chemical. The use of herbicides is often required in integrated weed control programs, but their level of use and time of application are
carefully determined. The judicious application of herbicides has become an integral part of agriculture, despite the emergence in recent years of compounds of greatly increased efficacy and safety (Huppatz, 1990). Naturally occurring bioactive compounds, particularly those shown to be non-toxic, would be useful in integrated weed control programs. The growing awareness of the environmental and public health consequence of agricultural chemical use has stimulated interest in the search for new, environmentally safe herbicides (Lydon and Duke, 1987).

**Naturally Occurring Inhibitory Compounds for Weed Control**

The use of natural products as the basis for new insecticides has been extremely successful. Many workers have isolated insecticidal, chemosterilizing, antileukemic, and antifungal substances from plants. However, only a few studies indicate their use as herbicides (Lydon and Duke, 1987).

A microbial fermentation product, bialaphos, is being marketed as a herbicide in Japan for control of grasses in rice fields (Lydon and Duke, 1987). The potential for use of compounds isolated from diverse sources, ranging from microbial to higher plants, has received increasing attention. These natural products often are safe materials and less likely to cause any concern of possible contamination of ground water from runoff or soil movement. So they have the requisite specificities to be ideal herbicides (Lax et al., 1988).

Rizvi *et al.* (1980) indicated that pesticides from plant sources have been shown to be less phytotoxic, more systemic, and more easily biodegradable than the synthetic ones. Use of natural compounds as herbicides, or as the chemical
basis for the development of new herbicides, offers several advantages. The wide array of phytotoxic compounds produced by plants provide many complex chemical structures that are unlikely to be found in the traditional synthesis strategies used by pesticide companies. Halogenated hydrocarbons, compounds of particular environmental concern, constitute about 60% of registered herbicides. Few natural compounds are halogenated. The majority of natural compounds from plants pose little health threat and degradation of natural compounds in the environment proceeds faster than that of traditional synthetic herbicides. These materials have the potential to reduce ground water contamination, and they can probably be classified as environmentally safe (Lydon and Duke, 1987).

Allelopathy

Allelopathy (literally, 'mutual harm') is conventionally regarded as describing chemical interactions between plants. Allelochemicals are the compounds that are involved in allelopathy (Lydon and Duke, 1987). Reese (1979) defined allelochemical as a 'nonnutritional chemicals produced by one organism that affect the growth, health and behaviour or population biology of other species'. Numerous studies have reported the allelopathic effects on weeds of extracts or residues from various plant species (Rice, 1984).

Dzyubenko and Petrenko (1970) reported that root excretions of the crop plants, *Lupinus albus* and *Zea mays*, inhibited growth of the two weed species, *Chenopodium album* and *Amaranthus retroflexus*, in laboratory and greenhouse experiments with water and sand culture. They also reported that the exudates of the roots of weed species stimulated growth of the cultivated
plants. It has also been reported that wheat, oats, peas, and buckwheat suppressed growth, accumulation of above-ground biomass, and leaf surface of lambsquarters (Neustruyeva and Dobretsova, 1972). In another study, the excretions from seed of hairy vetch have been tested against seed germination and seedling growth of thirteen species of weeds that were found to be markedly inhibited (Lazauskas and Balinevichiute, 1972).

Soybeans were found to have some ability to inhibit weed growth. Exudates from roots of selected soybeans suppressed velvetleaf enough to reduce plant dry weights by about 15%. Foxtail millet germination and dry weights were reduced 82% and 65%, respectively (Sommers, 1985). A screening test on 3000 accessions of the USDA world collection of *Avena* spp. germplasm was studied for their capacity to exude scopoletin, a naturally occurring compound shown to have root growth inhibiting properties (Fay and Duke, 1977). It was found that plants grown in close association with the toxic accession exhibited severe chlorosis, stunting, and twisting.

Allelopathy may legitimately be regarded as a component of biological control, by virtue of Lovett's (1991) definition that 'almost any process, occurring naturally or done artificially, which affects the relationship between organisms in such a way that the natural biological balance is restored, can be regarded as biocontrol'. Researchers have been studying the allelopathy phenomenon in hopes it may be turned against weeds and have suggested that allelopathy can be used in weed control in several other ways (Sommers, 1985). One method is to use allelochemicals from various sources as herbicides as alternative methods for weed control (Rice, 1984). There is potential for
allelochemicals to be developed as 'natural pesticides', a form of biological control.

Possible uses of plant derived compounds in biological weed control

Some natural plant compounds that are observed to inhibit growth and development of other plants may function as herbicides or serve as the starting point for chemical synthesis of biodegradable herbicides. Much interest has centered on the use of plant derived compounds as natural herbicides and they are considered to represent an environmentally sound approach to weed control (Rice, 1984; Lax et al., 1988).

The activity against a forb, Brassica hirta, and a grass, Panicum miliaceu, was investigated by screening 526 accessions of cucumber (Cucumis sativis) and 12 accessions of eight related Cucumis species, representing 41 nations of origin. It was found that incorporating the allelopathic characteristics of these crop cultivars into the cropping system, provided a means of gaining a competitive advantage over certain important weeds (Putnam and Duke, 1974).

Rhizobitoxine, produced by certain strains of Rhizobium japonicum in soybean nodules, was compared with 2 commercial herbicides, amitrole and metflurazone for its herbicidal effects. It was found that rhizobitoxine was equal in phytotoxicity to amitrol and more phytotoxic than metflurazone on a weight basis. It was potentially effective as a herbicide in amounts of 3 oz/acre (Owens, 1973).

Rizvi et al. (1980) extracted leaves and seeds from different plants and tested their herbicidal properties. They first started a survey of the herbicidal
activity of ethanolic extracts of leaves and seeds of over 50 plant species against seed germination of *Amaranthus spinosus*. They found that extracts of coffee (*Coffea arabica*) were the most inhibitory. Extracts of seeds or leaves of 12 other species caused 30 to 50% inhibition, and eight other species caused 20 to 30% inhibition at the same concentrations. The seed extract of *Coffea arabica* was fractionated in different organic solvents. All the fractions were tested for the desired activity and it was found that the chloroform fraction completely inhibited the seed germination of the test weed at 5,000 ppm. The same material had no effect on the germination and subsequent growth of *Phaseolus mungo*. They suggested that the fraction can be a possible source of natural herbicide. The most active phytotoxic compound in the chloroform fraction was identified as 1,3,7-trimethylxanthine (caffeine). Due to its selective nature, non-toxicity to the crop plants, and broad range of herbicidal activity, they suggested that it could be used as an effective natural herbicide in some crops.

Tentoxin, a cyclic tetrapeptide, cyclo [leucyl-N-methyl\(^2\)dehydrophenylglycyl-N-methyalanyl], is produced by the fungus *Alternaria alternata* (= *A. tenuis*). It has been reported to cause chlorosis in a variety of weeds in soybean and corn while not affecting these crops. Tentoxin-induced-chlorosis was observed in Johnsongrass [*Sorghum Balepense* (L.)Pers.], ivyleaf [*Ipomoea bederacea* (L.)Jacq.], and sicklepod (*Cassia obtusifolia* L.). Corn and soybean were insensitive to the toxin (Lax et al., 1988).

Although there are many distinct advantages of using safe materials as natural herbicides for weed suppression in integrated weed control systems, more work is needed on the isolation of plant derived, natural herbicides.
Because it is difficult to isolate sufficient quantities of the active compounds for whole plant testing, field data on these materials is limited (Lydon and Duke, 1987). One of the most investigated examples using field testing was on a leguminous plant, *Cassia sericea*, that could exert effective control of parthenium weed in the field through apparent allelopathic activity (Joshi & Mahadevappa, 1986). Both plants were imported to India, where parthenium weed has colonised at least five milliimi hectares of land. The release of natural inhibitors from growing barley plants has been quantified and the activity of two chemicals, gramine and hordenine, were assessed in bioassay and in a hydroponic systems (Liu & Lovett, 1989). These compounds affected the growth of a test plant species, white mustard (*Sinapis alba*). Hordenine also affected the growth of larvae of the common armyworm (*Mythimna convecta*), an insect pest of barley, and a fungal pathogen (*Drechslera teres*).

Broadening perceptions in areas related to agricultural systems management, have increased the growing acceptance of the merit of alternative approaches in the pursuit of 'sustainability' during the late 1980s (Lovett, 1991). A worldwide search for new types of useful and highly active plant protection chemicals is in progress. Although hundreds of compounds, representing dozens of chemical classes, have now been identified and developed, the users of these products and society continue to call for more innovation in chemical and biological plant protection (Voss and Geissubühler, 1990). There is a continuing need to develop natural herbicides. In the 1990s and later, it seems likely that the use of naturally occurring inhibitory compounds will be recognised as an important contributor to the widening array of biological control strategies (Lovett, 1991).
Corn gluten meal as a natural preemergence herbicide

It was observed, that stand establishment of creeping bentgrass (*Agrostis palustris* Huds.) was inhibited by incorporating unprocessed corn (*Zea mays* L.) meal into the soil (Christians, 1993). Subsequently, in the study of the effects of corn starch, corn gluten meal, corn germ, corn seed fiber, and corn meal applied to the soil surface, it was found that the corn gluten meal had the highest inhibitory activity on creeping bentgrass. More field and greenhouse studies on the use of corn gluten meal demonstrated that this material contained bioactive compounds that inhibited root formation in several monocotyledonous and dicotyledonous species, including crabgrass (*Digitaria* spp.). Seeds that germinated in a soil media to which corn gluten meal was added formed normal shoots, but no roots. The seedlings quickly died as the media dried. The inhibition of root formation can be used to prevent the establishment of weeds such as crabgrass (*Digitaria* spp.) in turf areas and other plant systems. A patent on the use of corn gluten meal as a natural preemergence herbicide was issued in July of 1991 (Christians, 1991).

Corn gluten meal is capable of inhibiting root growth of germinating seed, while no damage is observed to plants that have formed a mature root system. Corn gluten meal contains approximately 10% nitrogen and can also be used as a natural fertilizer material. Repeated field trials showed no detrimental effect from the corn gluten meal on mature grass plants (Christians and Liu, 1992). The combination of a natural weed inhibiting compounds with a natural nitrogen source would indicate that corn gluten meal can be a useful natural preemergence herbicide and fertilizer material (Christians, 1991).
Corn Gluten Meal

Corn (*Zea mays* L.) is produced in abundance and is the most important commonly used livestock and poultry feed in the United States. Corn contains about 72% starch on a dry basis. It is one of the best sources of metabolized energy among the grains. The availability of corn and soybean meal as economical sources of energy and protein has played an important role in the growth and development of the livestock and poultry industries (Wright, 1987). Protein content and composition were examined by Boundy et al. (1967) in 3 types of corn, dent, waxy, and high-amylose, using selective extractions. In general, the proteins in corn have a relatively high percentages of glutamic acid, leucine, proline, alanine, and aspartic acid, but are low in lysine and tryptophan.

The primary use of corn is for animal feed and for valuable food and industrial products through processing. Normal dent corn is the predominant corn processed. The greatest amount is processed by wet milling, which is one of three major processes used to manufacture food products from corn (May, 1987). The wet milling process involves an initial water soak to soften the kernels. This is conducted under carefully controlled conditions of temperature, time, sulfur dioxide (SO2) concentration, lactic acid content, etc. Steeping softens the kernels that are then milled into components separated by screening, centrifuging, and washing. Corn starch and nutritive sweeteners, corn oil, defatted corn germ meal, corn hulls (fiber), condensed fermented corn extractives (steep liquor), and corn gluten (the protein fraction) are produced by this process. The condensed fermented corn extractives, corn germ meal, and corn hulls, may be processed and sold as separate products or combined in
proper proportions and become corn gluten feed. A study to evaluate the coefficient of true digestibility and biological value of the by-products of corn in the wet milling process suggested that corn gluten and corn germ have a higher biological value than the steep water concentrate (Schulz, 1949).

A thick yellow slurry of corn gluten liquid containing 15 to 20% solids separated from starch by centrifuges give a stream containing 69-72% total protein on a dry substance basis. Conventionally, corn gluten liquid is filtered and dried in direct-fired, flash or rotary dryers to 10-12% moisture to produce solid corn gluten meal (CGM) which is sold as an animal feed product. Dried gluten meal should have a minimum of 60% protein and be finer than 12 U.S. mesh. No more than 20% should pass through a 100 U.S. mesh screen. Corn gluten meal is a mixture of protein, lipid, carbohydrate and ash, rich in xanthophylls which is important to poultry feeders (Hardwick and Glatz, 1989). Its composition can vary but commonly contains about 60% protein (Table 1). As the dehydrated protein stream resulting from starch separation, CGM is a good source of sulfur-containing amino acids, methionine and cystine, but very low in lysine and tryptophan (Table 2). The major protein fractions in corn gluten meal, zein (68%), glutelin (28%), and globulins (1.2%) are insoluble in H2O (Hardwick and Glatz, 1989).

The water insolubility renders CGM difficult to apply as an herbicide. This is undesirable for several reasons. First of all, herbicides are commonly applied over large plots of soil such as a farm field. Insoluble materials cannot be dissolved and sprayed. They are difficult to apply evenly. As a result, the
soil on which the herbicide is applied are not completely covered and therefore the effectiveness may be reduced. Moreover, water-insoluble or slightly soluble materials do not permeate the soil as well as do water-soluble materials. This may result in the reduction of herbicidal effectiveness.

Table 1. Proximate analysis of corn\textsuperscript{a} and corn gluten meal\textsuperscript{b}.

<table>
<thead>
<tr>
<th>Item</th>
<th>Corn (%)</th>
<th>Corn Gluten Meal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>As is</td>
<td>DSB\textsuperscript{c}</td>
</tr>
<tr>
<td>Moisture</td>
<td>15.0</td>
<td>---</td>
</tr>
<tr>
<td>Protein (Nx6.25)</td>
<td>8.0</td>
<td>9.5</td>
</tr>
<tr>
<td>Fat</td>
<td>3.6</td>
<td>4.3</td>
</tr>
<tr>
<td>Fiber</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ash</td>
<td>1.2</td>
<td>1.4</td>
</tr>
<tr>
<td>NFE\textsuperscript{d}</td>
<td>69.2</td>
<td>81.9</td>
</tr>
<tr>
<td>Starch</td>
<td>60.6</td>
<td>71.7</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Source: Anonymous (1982b).
\textsuperscript{b}Source: Anonymous (1982a).
\textsuperscript{c}Dry substance basis.
\textsuperscript{d}Nitrogen-free extract.
Table 2. Typical amino acid content of com and com gluten meal:

<table>
<thead>
<tr>
<th>Item</th>
<th>Corn</th>
<th>Corn Gluten Meal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>0.78</td>
<td>5.8</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.42</td>
<td>2.1</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.68</td>
<td>4.0</td>
</tr>
<tr>
<td>Cystine</td>
<td>0.19</td>
<td>1.2</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>1.77</td>
<td>15.3</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.37</td>
<td>1.8</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.25</td>
<td>1.3</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.34</td>
<td>2.6</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.05</td>
<td>11.1</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.22</td>
<td>1.1</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.15</td>
<td>2.1</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.42</td>
<td>4.2</td>
</tr>
<tr>
<td>Proline</td>
<td>0.84</td>
<td>6.1</td>
</tr>
<tr>
<td>Serine</td>
<td>0.44</td>
<td>3.4</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.31</td>
<td>2.2</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.07</td>
<td>0.3</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.33</td>
<td>3.2</td>
</tr>
<tr>
<td>Valine</td>
<td>0.38</td>
<td>3.0</td>
</tr>
</tbody>
</table>

aSource: Anonymous (1982a).

bDry substance basis.
Methods of Isolation and Identification of Peptides

A specific protocol for the proof of a biologically active chemical is suggested by Putnam and Tang (1986). It generally involves the following sequence of studies. First of all, it is necessary to demonstrate interference using a suitable control, to describe the symptomology, and to quantitate the growth reduction. The next step is to isolate, characterize, and assay the chemicals against species that were previously affected. This is followed by obtaining toxicity with similar symptomology when chemical(s) are added back to the system. Numerous studies in the area of separation and identification of naturally occurring compounds have been documented.

It has been found that aparagus (*Asparagus officinalis* L.) roots contain autotoxic and allelopathically active compounds (Hezebroek *et al.*, 1989). An allelochemical collected by a cellulose column chromatography method from the extract of asparagus fresh root tissue was identified as caffeic acid by melting point, thin-layer chromatography and infrared spectrum analysis (Miller *et al.*, 1991).

Quackgrass (*Agropyron repens* L. Beauv.) is a highly competitive perennial grass weed. It has been shown to contain compounds that are able to inhibit germination and seedling growth of many plant species (Ohman and Kommedahl, 1964; Toai and Linscott, 1979; Weston and Putnam, 1986). The compounds responsible for the phytotoxicity in quackgrass were evaluated using bioassays of seedling growth of corn (*Zea mays* L.) and bean (*Phaseolus vulgaris* L.). They were identified as 5-hydroxyindole-3-acetic acid (5-HIAA) and 5-hydroxytryptophan (5-HTP). This was done by thin layer chromatography, high performance liquid chromatography (HPLC), mass
spectrophotometry, UV spectrophotometry, IR spectrophotometry, and C,H,N analysis.

Residues and aqueous extracts of rye (Secale cereale L. ‘Wheeler’) shoot herbage have been shown to be phytotoxic to several plant species (Barnes, Putnam, 1985). The most active compounds identified by cress (Lepidium sativum L. ‘Curly’) root growth assay, were separated in diethyl ether by sequential partitioning of aqueous extract rye shoot against a series of solvents of increasing polarity. The compounds were identified as 2,4-dihydroxy-1,4(2H)-benzoxazin-3-one (DIBOA) and a breakdown product 2(3H)-benzoxazolinone (BOA). DIBOA is a significant inhibitory material on barnyardgrass (Echinochloa crus-galli L. Beauv.) (Barnes et al., 1987).

It has been reported that American cranberry (Vaccinium Macrocarpon) plants produce germination and growth inhibitors of wheat. Parasorbic acid, a known growth inhibitor, in the form of its glucoside was isolated and identified from the extract of cranberry leaves and its inhibitory activity was examined using wheat seed (Triticum aestivum var. “Olaf” spring wheat) bioassay (Wepplo, 1987).

The leaves and seeds of Lantana camara L., one of the world’s worst weeds, were found to be toxic to many animals (McSweeny and Pass, 1982; Sharma et al., 1981) and humans (Mortan, 1971). Numerous studies reported that lantana extracts were phytotoxic to various weed and crop species (Pope et al., 1983; Rice, 1984; Achhireddy and Singh, 1984; Achhireddy et al., 1985; and Mersie and Singh, 1987). Phytotoxic compounds were fractionated from crude aqueous extracts of lantana leaves and evaluated by bioassay of ryegrass (Lolium multiflorum Lam.) germination and seedling growth. An HPLC
method was used to identify 13 phenolic compounds responsible for the phytotoxicity to ryegrass seedling. Radicle elongation was found to be more sensitive to the toxins than shoot elongation (Singh, et al., 1989).

Rice (1984) classified the many thousands of secondary compounds produced by higher plants or microorganism into 14 categories. They are listed as follows; water-soluble organic acids, straight chain alcohols, aliphatic aldehydes, and ketones; simple unsaturated lactones; long-chain fatty acids and polyacetylenes; naphthoquinones, anthraquinones and complex quinones; phloroglucinol and polyphloroglucinols; cinnamic acid derivatives; coumarins; flavonoids; condensed or hydrolyzable tannins; terpenoids and steroids; amino acids and polypeptides; alkaloids and cyanohydrins; sulfides and mustard oil glycosides; purines and nucleosides. Most identified naturally inhibitory compounds fit in one of the fourteen chemical categories with a few exceptions (Rice, 1984). However, there are numerous unidentified inhibitors that may be exploited as possible natural herbicides.

Amino acids and polypeptides are one of the 14 categories grouped by Rice (1984). Since corn gluten meal is the protein fraction of the corn from the wet milling process, the active component(s) may be amino acids or peptide related materials. Proteins or polypeptides are unique classes of molecules consisting of amino acids as the building blocks. Amino acids can be divided into two major groups. One group consists of 20 naturally occurring amino acids found in all living systems as free amino acids, as peptides, or as proteins. The other group occurs in a limited number of organisms and is not found in proteins (Bailey, 1990). The side-chains are categorized according to their polarity, nonpolar or hydrophobic vs. polar or hydrophilic.
Peptides are the polymers of two or more amino acid molecules connected by amide bonds, peptide (C-N) linkages (Robinson, 1983). Peptides have an amino group at one end, a carboxylic acid group at the other end, and side-chains separated by amide bonds. The properties of peptides and proteins, solubility, structure, and function, are characteristic of their amino acid composition and sequence (Hodges and Mant, 1991). The three factors that determine the physical properties of amino acids and peptides are the net charge at any given pH, the pH at which there was no net charge (isoelectric point, pI), and the hydrophilicity or lipophilicity of the side-chains (Bailey, 1990). The hydrophobicity and hydrophilicity as well as the number of charged groups present are important factors in the separation of peptides and proteins.

Although peptides are constructed from relatively simple building blocks, they are found to exhibit a remarkable range of biological properties. They can act as antibiotics, hormones, food additives, poisons, or pain-killers. Because of their medicinal properties, the study of peptides has become one of the most active areas of current research (Bailey, 1990). Some polypeptides have been identified as having inhibitory activity. Owens (1969) reported that lycomarasmin, produced by *Fusarium oxysporum f. lycopersicum*, could cause wilting of tomato cuttings. Victorin, produced by *Helminthosporium victoriae*, caused blight in Victoria oats. Carbtoxinine, produced by *H. Carbonum*, and toxins A and B, produced by *Periconia circinata*, could cause milo disease in certain grain sorghums. Rhizobitoxine, a noncommon amino acid, inhibits the conversion of methionine into ethylene by irreversibly inactivated β-cystathionase. It was identified as 2-amino-4-(2-amino-3-hydroxypropoxy)-trans-but-3-enoic acid (Owens *et al.*, 1972).
Although the art of separation is something of a mystery, there are many principles governing the discipline. Besides physical properties, the size molecular weight of peptide and can be used in the purification process (Bailey, 1990). The first step in the separation, is to obtain crude extracts from selected samples (Cutler, 1986). The objectives for sample preparation are to obtain the component of interest in solution, free from interfering matrix elements, and in appropriate concentration for detection or measurement. Solid phase extraction (SPE) recently has become one of the fastest growing sample preparation and cleanup techniques (Majors, 1993). The SPE procedure is based on a specific molecular interaction yielding excellent recoveries, unlike liquid-liquid extraction that often results in variable sample recovery. Some advantages of using SPE are that it is fast, versatile, and selective. It also uses less organic solvents than other sample preparation techniques, such as liquid-liquid extraction.

Gel filtration uses a polymeric support that has a relatively open structure and separates compounds on the basis of their size (Bailey, 1990). Usually, the mixture of peptide and proteins is loaded onto the top of a column of porous polymer that is then eluted with a proper solvent or buffered water. Separation by gel filtration depends on the differing abilities of the various sample molecules to enter pores. Molecules larger than the molecular weight cutoff (MWCO) are unable to enter the pores and will come off the column first. The smaller molecules can enter the pore in the polymer and spend a proportion of time in the stationary phase. They are eluted in order of decreasing molecular size. Results in gel filtration are typically expressed in
the form of an elution diagram showing the variation of solute concentration in
the eluent with the volume of eluent passing through the column.

Peptides can possess a positive or negative net charge at any pH. This net
charge is greatly influenced by the nature of the side-chains of their constituent
amino acids (Bailey, 1990). Typical ion exchange resins can be either positively
or negatively charged polymers. A solution of a mixture of peptides passed
down a column containing a charged polymeric support will elute at different
rates, depending mainly on the net charge of the peptides. Negatively charged
peptides have a high affinity for an anion exchange resin, while positively
charged peptides will be eluted rapidly. To elute the negatively charged
peptides off the column with good resolution, within a reasonable time span,
the concentration of eluent is varied with time.

High performance liquid chromatography (HPLC) has become the fastest
growing analytical technique since it was developed around 1969. It is a
technique that utilizes the basic liquid chromatography principles but allows
separation of chemicals faster and with greater resolution than conventional
chromatography techniques. Small diameter packing materials with a high
surface area are used as supports in the HPLC column. These packing
materials can withstand high pressure in order that the solvent can be passed
through the column. ‘High performance’ refers to the quality of the separations
that can be achieved. Since HPLC is not limited to sample volatility and
thermal stability as is gas chromatography, nearly all classes of organic
compounds can be separated by this technique (Runser, 1981). Modern
supports as stationary phase are composed of extremely fine particles to
increase the effective surface area of the support; they can be ion exchange, gel filtration, silica or reversed phase.

Reversed-phase HPLC (RPC) is a particularly effective method of purifying peptides (Bailey, 1990). In RPC, non-polar stationary phases (lipophilic alkyl groups attached to the support, eg. C18-RP) and polar mobile phase are employed, and samples are separated by their hydro-phobicity. The sample running down the column in a polar solvent can be continually partitioned between a hydrocarbon stationary phase and the more polar mobile phase. Therefore, hydrophilic peptides are eluted rapidly, whereas lipophilic ones have longer retention times. Numerous studies of the influence of the mobile phase and the role of packing in reversed-phase HPLC, have resulted in it becoming a mature and well-established technique in the isolation of peptides and proteins (Esser and Unger, 1991).

Duvick et al. (1992) isolated several small, acid soluble, basic peptides with antimicrobial activity from corn kernels. One of these peptides (MBP-1) was purified to homogeneity by a combination of solvent extraction, cation exchange chromatography, and gel electrophoresis. The peptide had a molecular weight of 4127.08 as determined by plasma desorption mass spectroscopy. Its amino acid composition was determined by a modified amino acid analysis method, and its primary sequence was determined by Edman degradation. A synthetic MBP-1 was prepared and tested to be as antifungal as the naturally occurring peptide.

There is no single purification method that allows for the isolation of all chemicals. In general, a method that would recover large quantities of materials, but give rather crude purification, is used initially (Bailey, 1990). As
the amount of sample gets smaller, the higher-resolution chromatographic techniques become important. Most purification protocols utilize a combination of separation steps to achieve isolation.

There are three different types of detection that can be used during the purification of peptides. They include non-destructive, destructive chemical, and biochemical methods. Within non-destructive methods, monitoring the UV absorption of the eluent is the most commonly used method for detecting peptides eluted from a chromatography. Because there is always a λ_{max} at about 215 nm, monitoring a wavelength on the shoulder of this absorption is usually practiced (Bailey, 1990). If the peptide contains aromatic side-chain, phenylalanine or tyrosine, absorption at 280 nm can be monitored.

Amino acid analysis (AAA) is used to determine which amino acid residues are present in an isolated peptide or protein. The AAA provides an important quantitative parameter in the characterization of isolated peptide or protein samples. Recent improvements in instruments and methodology enable the determination of amino acids in the picomole range. It is usually done by first subjecting samples to total acidic hydrolysis that can liberate the constituent amino acids. The most common hydrolysis method uses 6 N HCl for 20 to 24 h at 110°C under vacuum (Ozols, 1990).

Currently, about one-half of the amino acid analyzers employ ion-exchange separation of the free amino acids, followed by postcolumn derivatization with ninhydrin, o-phthalaldehyde, or fluorescamine. The other half of the amino acid analyzers use precolumn derivatization with phenyl isothiocyanate (PITC), followed by reversed-phase high-performance liquid chromatography (HPLC) separation of the resulting phenylthiocarbamyl (PTC)
amino acids (Ozols, 1990). The use of on-line HPLC systems employ microbore columns for phenylthiohydantoin (PTH) amino acid detection. The AAA of PTC derivatives by HPLC analyzers is about five times as sensitive as the ion-exchange resolutions. By comparing with standard amino acids, the amounts of each amino acid can be determined by the area of each peak (integration) and confirmation of the absence of unusual amino acids (Bailey, 1990).

Identification of the N-terminus of peptides and proteins that reveals the sequencing of the amino acid residues is important for characterizing bioactive peptides and the structural and functional domain of proteins. The N-terminal sequences of peptides and proteins are determined using repeated cycles, either manual or automated, of the Edman degradation reaction (Matsudaira, 1990). Each degradation cycle consists of three steps: coupling, cleavage, and conversion. The unmodified N-terminus of peptide or protein is modified with PITC under basic conditions to generate a PTC peptide derivative in the coupling step. The PTC-N-terminal residue is cleaved from the peptide by either liquid or gaseous trifluoroacetic acid (TFA) to form an anilinothiazoline (ATZ) amino acid derivative of the original N-terminal residue. In the conversion step, the unstable ATZ-amino acid is converted by acid to PTH-amino acid, which is more stable. During one cycle of the Edman reaction, the N-terminal residue removed from the polypeptide is identified by HPLC. The N-terminal residue of the (n-1) polypeptide is available for another cycle of the Edman reaction. The PTH-amino acid generated during each sequencing cycle is identified by UV absorbance and HPLC chromatography with a reversed-phase HPLC support. With the automated gas-phase sequenator, the sample is adsorbed to a polybrene-coated glass-fiber disk or is electroeluted onto a
specially treated glass-fiber filter or a porous polyvinylidene difluoride membrane. Certain reagents used for the coupling and cleavage steps are delivered as gases. The peptide remains bound to the support during the coupling and cleavage steps, which occur in a temperature-controlled reaction chamber (Matsudaira, 1989).

Bioassays in the Study of Potential Natural Herbicides

Bioassays are necessary to evaluate the bioactive potential of plant derived inhibitors and to follow the activity during the extraction, purification, and identification process. They should be employed to determine the bioactivity of chemicals in every step in the procedure of isolating and identifying bioactive compounds (Leather and Einhellig, 1986).

The most common bioassay used to test for inhibitory activity of phytochemicals is the inhibition of seed germination. Leather and Einhellig (1986) summarized several studies describing this bioassay technique. However, they found little standardization for seed germination bioassays. There is little information on the source of bioactive phytochemicals, methods of extraction, and fraction concentration. There is also a need for the comparisons with known compounds having demonstrated bioactivity. Few difficulties were observed when the comparisons were made of plant extracts prepared with different solvents, at different concentrations, and when evaluations were made with different species of seeds (Stowe, 1979). A seed-germination bioassay is usually conducted in petri dishes by placing the selected species of seed on a supporting medium saturated with the test solution. The petri dishes containing seeds and test solutions are incubated
within a controlled environment chamber with a light and dark cycle, with temperature regimes optimum for germination of the selected seed. Germination data most often expressed as germination percentages, are taken after incubation for a certain length of time specific to each species. Germination that leads to the development of the embryo into a seedling, is usually defined by the protrusion of some part of the embryo from the seed coat. There is no general rule as to which part of the embryo first pierces the seed coat. For some seeds, it is the shoot that protrudes first. But in most seeds, it is the radicle that is first to protrude and therefore germination is frequently equated with root protrusion (Mayer and Poljakoff-Mayber, 1975). In some studies, germination was defined as the emergence of the radicle exceeding 2 mm beyond the seed coat (Leather and Einhellig, 1986).

Plant growth and development bioassays were employed in addition to the germination bioassays in numerous studies reviewed by Leather and Einhellig (1986). The phytoactivity was demonstrated using radicle and hypocotyl/coeleoptile elongation along with germination percentages. However, many of them lacked sufficient replications in their experiment designs because of the requirement of the tedious measurements of radicle and hypocotyl/coeleoptile elongation. The measurement of radicle elongation is complicated by curling and other alterations in morphology when seeds are germinated in petri dishes. Some investigators used modified methods for the rapid measurements in growth and development bioassays (Leather and Einhellig, 1986).

Besides radicle and hypocotyl/coeleoptile elongation, tissue elongation, fresh and dry weights, seedling height, and root/shoot ratios have been
measured in growth and development assays. In a study evaluating the effects of allelopathic compounds and their analogs on various growth parameter (GP), Shilling and Yoshikawa (1987) found root length and root fresh weight were the most sensitive parameters. Because these measurements were extremely time consuming, they developed a rapid seedling bioassay that used predicted shoot-plus-root fresh weight models. This allowed for the evaluation of the biological activity of allelochemicals qualitatively and quantitatively during the purification of compounds.

In general, seedling growth bioassays were more sensitive than germination bioassays (Leather and Einhellig, 1985). For instance, in a study of the inhibition of grain sorghum seedlings, involving the use of several cinnamic acids, the threshold for inhibition of seedling growth was about 1/10th that of the level found for radicle elongation and 1/25th of the level for inhibition of germination (Einhellig et al., 1982).

One of the disadvantages of using bioassays to follow bioactivity is the requirement of a large amount of sample. This is particularly difficult in the study of purification and identification of bioactive compounds when only small amounts of sample are available during fractionation of the plant extract (Leather and Einhellig, 1985). Another shortcoming often stated is the time required in bioassay for plant growth. However, the use of a bioassay in the study of bioactive compounds is the only reliable method of tracing these materials in the isolation process.
MATERIALS AND METHODS

Selection of a Water Soluble Sample from Corn Gluten Meal

Thirty different samples derived from corn gluten meal or other grain materials (Table 3) were obtained from Grain Processing Company of Muscatine, Iowa. A number was assigned to each sample according to the order of arrival. Samples were tested in controlled environments both in the greenhouse and growth chambers for their inhibitory effects on test plants. From four separate studies, one sample with the greatest inhibitory activity was chosen for the isolation and identification of the bioactive compound(s).

Study 1

In the first study, corn gluten meal related samples numbered #1 to #12 were used to study the effects on germinating seeds under controlled environmental conditions (Table 3).

Smooth crabgrass (*Digitaria ischaemum* Schreb.) was the test species in the greenhouse bioassays. Plastic pots were filled with a Nicolett soil with a pH of 7.5 (fine-loamy mixed mesic Aquic Hapludoll) with a 58 cm\(^2\) surface area and a 5 cm depth. Seeds of crabgrass weighing 0.11 g were spread evenly on top of the soil. Preweighed, dry samples of the 12 test materials were applied uniformly on the soil surface at rates of 0, 0.86, 1.72, 3.44, and 6.88 g/dm\(^2\), except sample #8 that did not have enough material for the 3.44 and 6.88 g/dm\(^2\) rates. The study was replicated three times except for samples for
Table 3. List of samples derived from corn gluten meal and other crop materials

<table>
<thead>
<tr>
<th>Designated Sample #</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Study 1</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Corn Gluten Meal, Flash Dried</td>
</tr>
<tr>
<td>2</td>
<td>Gluten Meal Treated with Bacterial Proteinase, Flash Dried</td>
</tr>
<tr>
<td>3</td>
<td>Gluten Meal Treated with Fungal Proteinase, Flash Dried</td>
</tr>
<tr>
<td>4</td>
<td>Gluten Meal Treated with Bacterial and Fungal Proteinases</td>
</tr>
<tr>
<td>5</td>
<td>Gluten Solution Treated with Bacterial Proteinase, Flash Dried</td>
</tr>
<tr>
<td>6</td>
<td>Gluten Filtrate</td>
</tr>
<tr>
<td>7</td>
<td>Alcohol Soluble Fraction of Gluten Filtrate</td>
</tr>
<tr>
<td>8</td>
<td>Alcohol Insoluble Fraction of Gluten Filtrate</td>
</tr>
<tr>
<td>9</td>
<td>Gluten Filtrate Treated with Cation Resin</td>
</tr>
<tr>
<td>10</td>
<td>Gluten Solution Treated with Bacterial Proteinase, Freeze Dried</td>
</tr>
<tr>
<td>11</td>
<td>Gluten Solution, Drum Dried</td>
</tr>
<tr>
<td>12</td>
<td>Gluten Solution Treated with Bacterial Proteinase, Drum Dried</td>
</tr>
<tr>
<td><strong>Study 2</strong></td>
<td></td>
</tr>
<tr>
<td>13 (= 1)</td>
<td>Gluten Meal Mixed with Corn Hulls and Germs, Flash Dried</td>
</tr>
<tr>
<td>14</td>
<td>Gluten Meal without Corn Hulls and Germs, Flash Dried</td>
</tr>
<tr>
<td>15 (= 5)</td>
<td>Gluten Hydrolysate from Gluten Solution, Flash Dried</td>
</tr>
<tr>
<td>16</td>
<td>Gluten Hydrolysate Totally Ion Exchanged</td>
</tr>
<tr>
<td>17</td>
<td>Soluble Corn Steep Liquor Solids</td>
</tr>
<tr>
<td>18</td>
<td>Insoluble Corn Steep Liquor Solids</td>
</tr>
<tr>
<td><strong>Study 3</strong></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Wheat Gluten</td>
</tr>
<tr>
<td>20</td>
<td>Wheat Gluten Hydrolysate</td>
</tr>
<tr>
<td>21</td>
<td>-30 Mesh Gluten Filter Cake</td>
</tr>
<tr>
<td>22</td>
<td>-30 &amp; +12 Mixed Mesh Gluten Filter Cake</td>
</tr>
<tr>
<td>23</td>
<td>+12 Mesh Gluten Filter Cake</td>
</tr>
<tr>
<td>24 (= 15)</td>
<td>Corn Gluten Hydrolysate</td>
</tr>
<tr>
<td>25</td>
<td>Soybean Meal</td>
</tr>
<tr>
<td><strong>Study 4</strong></td>
<td></td>
</tr>
<tr>
<td>26 (= 24)</td>
<td>Corn Gluten Hydrolysate</td>
</tr>
<tr>
<td>27</td>
<td>Precipitate from #26 (Gluten Hydrolysate)</td>
</tr>
<tr>
<td>28</td>
<td>Gluten Hydrolysate Filtrate</td>
</tr>
<tr>
<td>29</td>
<td>Carbon Treated Gluten Hydrolysate Filtrate</td>
</tr>
<tr>
<td>30</td>
<td>Ion Exchanged &amp; Carbon Treated Gluten Hydrolysate Filtrate</td>
</tr>
</tbody>
</table>
which there was insufficient material. Sample #6 had only 2 replicates at 0.86, and 1.72 g/dm² and only one replicate for the 3.44 and 6.88 g/dm² treatments. Sample #7 at 1.72, 3.44 and 6.88 g/dm² and sample #8 at 1.72 g/dm² had only 2 replicates. Sample #9 was replicated 2 times at 3.44 g/dm² and only one time at 6.88 g/dm². Sample #11 had one replicate at 6.88 g/dm² treatment. A completely randomized experimental design was used. All pots were placed randomly first on the mist bench where constant moisture was available for 6 days. Following germination, the pots were moved to a greenhouse bench, and watered every 2 to 3 days using a fine mist nozzle. The greenhouse temperature was maintained in a range of 18 to 32°C.

Data were collected on the number of live grass shoots at 18 days-after-seeding (DAS). Fresh clipping weights were taken at 35 DAS. These two measurements from each pot were averaged for each treatment.

Smooth crabgrass and creeping bentgrass (*Agrostis palustris* Huds.) were used for the petri dish bioassays. The 12 test samples were applied at rates of 0, 0.118, 0.236, 0.355, and 0.473 g/dm² to two layers of blotter paper measuring 42.25 cm². Before the treatment, the blotter paper was placed in a 9-cm diameter petri dish (Fisher Scientific Co., Pittsburgh, PA) and moistened with 7 mL deionized distilled water (D. D. H₂O). Eighteen smooth crabgrass seeds were placed on the blotter paper. The dishes were covered with a lid and sealed with parafilm. They were incubated in a growth chamber at a day/night temperature of 25 ± 0.5°C. A 16 h photoperiod was used and light levels were maintained at 70 μmol/sec/m² with fluorescent light. After 21 days, the germination rate expressed in percentage was derived by dividing the number
of germinated seeds by 18, the number of seeds used in each plate, and multiplying by 100.

Study 2

In the second study, the effects of six corn gluten meal derived samples numbered #13 to #18 were bioassayed using creeping bentgrass and perennial ryegrass (*Lolium perenne*) (Table 3). Plastic pots filled with the same type of soil as in study 1 were used for the bioassays. Creeping bentgrass was seeded on the 64-cm$^2$ surface area soil at a rate of 0.16 g/dm$^2$. Sample materials were applied at rates of 0, 0.78, 1.56, 3.13, 4.69, 6.25, and 9.38 g/dm$^2$ for all samples except for sample #18, which had only enough material for 3 levels of application; 0.78, 1.56 and 3.13 g/dm$^2$. All 38 pots were randomly placed in the greenhouse after 6 days on the mist bench; the environments and irrigation method were maintained with the same conditions as in study 1. Data were taken at 28 DAS on average plant height and fresh clipping weight of live plants.

Perennial ryegrass was seeded at 1.56 g/dm$^2$. The same seven rates used in the creeping bentgrass bioassay described above of samples #13, 14, 15 and 16 were used. Sample #17 and #18 were not tested because there were no materials available. The study was conducted as previously described. Data were collected as fresh clipping weight at 27 DAS.

Perennial ryegrass seeds were also used to test the bioactivity of samples, #13, 14, 15, 16, 17, and 18 in a petri dish bioassay. A volume of 6 mL of D. D. H$_2$O was applied to 2 layers of blotter paper measuring 36 cm$^2$. The test materials were applied at 0, 0.028, 0.056, 0.139, 0.222, 0.278, 0.417, and
0.556 g/dm² to the surface of the blotter paper in the petri dishes. Sixteen perennial ryegrass seeds were placed on the treated blotter paper. All 48 dishes were randomly placed in a growth chamber. Irradiance was maintained at 70 μmol/sec/m² using fluorescent light. A day/night temperature of 25/15°C and a 16 h photoperiod were established. The number of normal seedlings that had roots length greater than 0.5 cm were counted at 14 DAS.

Study 3

In the third study, perennial ryegrass was used to investigate the effects of the samples #16, 19, 20, 23, 24, and 25 on seedling growth in the greenhouse (Table 3). The same type of soil as described in study 1 was used. Perennial ryegrass was seeded at 0.78 g/dm² on 64 cm² plastic pots. The six test samples were evaluated at the rates of 0, 0.78, 1.56, 3.13, 4.69, 6.25, and 9.38 g/dm². The control was replicated 7 times but the test samples were not replicated because of the lack of available materials. Pots were kept in the greenhouse and watered regularly in the same manner as described in study 1. Data were collected on the average plant height and fresh clipping weight of each treatment at 28 DAS.

Samples of #19, 20, 21, and 22 (Table 3) were tested in petri dish bioassays at rates of 0, 0.028, 0.056, 0.139, 0.222, 0.278, 0.417, and 0.556 g/dm². The dry samples were applied to the top of 2 layers of 36 cm² blotter paper that was presoaked with 6 mL of D.D. H₂O. Sixteen perennial ryegrass seeds were placed on the blotter paper and the dishes were sealed with parafilm. All sealed dishes, including 6 controls, were incubated for 15 days in the growth chamber under the same conditions as in the previous evaluation.
Data were collected on the number of normal seedlings with root growth greater than 0.5 cm at 15 DAS.

**Study 4**

Based on the results from 3 previous studies, the bacterial proteinase hydrolysate (#5, 15, and 24) was found to be the most active sample among the corn gluten derived materials. In this study, four samples, #27, 28, 29, and 30, fractionated from the corn gluten hydrolysate, listed as #26 in Table 3, and some of the previously tested samples were evaluated for their inhibitory activity.

The greenhouse evaluation was conducted in the same way as described in study 3, except perennial ryegrass was seeded at 1.56 g/dm\(^2\). The pots were set up in the same manner as described above. Samples, #14, 21, 22, 26, 27, 28, 29, and 30, were applied at 0, 0.78, 1.56, 3.13, 4.69, 6.25, and 9.38 g/dm\(^2\). All treatments had one replicate except the control that had 8 replicates. The same location and time period described above were employed. Fresh clipping weight measurements were taken at 31 DAS.

In petri dishes, samples #26, 27, 28, 29, and 30 and sample #14 (Table 3) were tested at 0, 0.028, 0.056, 0.139, 0.222, 0.278, 0.417, and 0.556 g/dm\(^2\). Perennial ryegrass was the test species. The bioassay conditions were the same as described in study 3.

Alternatively, a dose response curve study of water soluble samples #26, 28, 29 and 30 was conducted using the perennial ryegrass bioassay that was modified as follows. A series of dilutions at 2.5, 5.0, 10, and 25 mg/mL of each sample was prepared. One ml of the test solution was applied to 1 layer of 7 cm
diameter Whatman No. 1 filter paper (Whatman International Ltd. Maidstone, England) measuring 38.5 cm$^2$, on which 10 perennial ryegrass seeds were placed. The petri dish was covered with a lid, sealed with parafilm and incubated in the growth chamber. The temperature was maintained at 25/15°C day/night. A 16 h photoperiod was used and the light intensity was maintained at 70 μmol/sec/m$^2$ with fluorescent lights (AOSA, 1988). After 14 days’ incubation, dishes were opened and seedlings were carefully removed from the filter paper using forceps. The lengths of the longest root and shoot were measured to the nearest mm. The data were expressed as a percentage of the control root length.

**Isolation and Identification of Bioactive Compound(s)**

**from Corn Gluten Hydrolysate**

Based on the results from phase I, it was found that the bacterial proteinase hydrolyzed corn gluten meal was the most effective in inhibiting seedling establishment of the 3 test species. The gluten hydrolysate could also provide a water-soluble material comprising one or more components that were much more active than the corn gluten meal itself in inhibiting root formation. Therefore, the corn gluten hydrolysate was chosen for isolation and identification of the inhibitory compound(s).

Because of the small amounts of bioactive materials available for testing in the isolation and identification phase, modifications were made in the bioassay technique. Ten perennial ryegrass (*Lolium perenne*) seeds were placed on 1 layer of 7 cm diameter Whatman No. 1 filter paper measuring 38.5 cm$^2$ in a 9-cm diameter petri dish. One ml of test solution was applied to each
petri dish. The dish was covered with a lid and sealed with parafilm and placed in a controlled environmental chamber. The light intensity in the growth chamber was maintained at 70 \( \mu \text{mol/sec/m}^2 \) with 25°C/15°C day/night temperature and a 16 h photoperiod. After 10 days, the longest root and shoot of each seedling in each plate were measured to the nearest millimeter, and the measurements of seedlings from the same dish were averaged. Seven out of the 10 seedlings in each petri dish, found to have root lengths within ± 1 standard deviation, were selected and averaged. The seven measurements of each treatment was divided by the control root length, multiplied by 100, and expressed as a percentage of control length (%). For quantitative purification purposes, the amount of material required to inhibit 50% of the control length was defined as 1 unit of activity.

Gluten hydrolysate powder was dissolved in D.D. H\(_2\)O to make a 10% solution. This aqueous solution of gluten hydrolysate was designated as GH1. A dilution series of GH1 was prepared for perennial ryegrass bioassay. The GH1 was filtered through a 0.2 \( \mu \text{m} \) membrane filter (Gelman Sciences Inc, Ann Arbor, MI). The filtrate was designated as GH7. A dilution series of GH7 was made for the perennial ryegrass bioassay.

A 28x998 mm (bed volume = 630 mL) Sephadex G-15 (Sigma Chemical Company, St. Louis, MO) gel filtration column was calibrated by loading a solution of 0.01 g of blue dextran and NaCl in 1 mL D.D. H\(_2\)O onto the column. The conductivity of effluent was measured using a YSI Model 31 conductivity bridge (YSI, Orangeburg, NY). Thirty mL of GH7 were loaded onto the column. The column was eluted with D.D. H\(_2\)O at a flow rate of 1.6 mL/min. The effluent (GH17) was collected in 80 tubes with 270 drops in each tube using a
Gilson Model 201 fraction collector (Gilson Medical Electronics, Inc., Middleton, WI). Selected tubes were bioassayed for the root-inhibiting bioactivity using the perennial ryegrass bioassay developed in this research as described above. Bioassay results showed that tubes #50 to 56 (#50GH17 to #56GH17) contained the root- and shoot-inhibiting compound(s). These tubes (GH17s) were freeze-dried and subjected to further purification steps.

Twelve mg of the #53GH17 were injected into a HPLC system (ISCO Inc., Lincoln, NE) with Dynamax C18-reversed phase (C18-RP), 250x10 mm I.D., 5 μ particle size, 100 Å pore size, column (Rainin 80-299-C5, Woburn, MA). A methanol (MeOH) in water linear gradient was used, with 0 to 25% MeOH in 50 minutes, 25 to 100% MeOH in the next 25 minutes, maintained at 100% MeOH for 15 minutes, and 100 to 0% MeOH in the next 10 minutes. The flow rate was 4 mL/min and the sample was monitored by UV absorbance at 280 nm with 0.02 AUFS (absorbance units of full scale). Fractions were collected at 10-minute intervals, for the first 50 minutes, and at a 25-minute interval for the next 50 minutes. These seven fractions were subjected to the perennial ryegrass bioassay to locate the root-inhibiting bioactivity. It was found that there were four active regions of the elution. The first region, that was eluted with 0-5% methanol in 10 minutes, was chosen for further purification.

The bioactive #53GH17 was also passed through C18 Sep-Pak cartridges (Waters Chromatography Division, Millipore Corporation, MA) and further fractionated. Three fractions were collected; GH27-1 which was not trapped by the cartridge, GH27-2 eluted by 10% MeOH, and GH27-4 eluted by 100% MeOH.
To examine the cleanup effect of the C18 Sep-Pak step, 5 mg of GH17 and 0.08 mg of GH27-1 were separately injected onto a Hibar C18-RP, 5 μ, 250x4.0 mm I.D., 5 μ, 100 Å column (EM Science, Gibbstown, NJ) on an HPLC (ISCO) with the same gradient as described above at a flow rate of 1 mL/min monitored at A280 with 0.05 and 0.02 AUFS, respectively.

Six mg of the GH27-1 were injected on to the C18-RP HPLC, 250x10 mm I.D. column (Rainin) with a MeOH in water linear gradient of from 0 to 5% in 10 min at a flow rate of 4 mL/min. The sample was monitored by UV absorbance at 214 nm with 1.0 AUFS. Fractions were collected based on the major peaks of the chromatogram. The same peaks were pooled from 6 consecutive runs for perennial ryegrass bioassay.

One peak (GH47-4) of the 10 peaks was identified as bioactive and subjected to amino acid analysis and peptide sequencing that were conducted by the Protein Facility at Iowa State University. Determination of the amino acid composition was performed with the Applied Biosystems Model 420A derivatizer, Model 130A separation system with 220x2.1 mm I.D., 5 μ PTC column, and a Model 920A data analysis system (Applied Biosystem, Inc., Foster City, CA).

The GH47-4 isolated by C18-RP HPLC was derivatized with phenylisothiocyanate (PITC) on an Applied Biosystem Model 420A derivatizer to form phenylthiocarbamyl (PTC) peptides that were separated using a C18-RP narrow-bore, 250x2.1 mm I.D. column (Vydac, Hesperia, CA). Seven peaks were collected and sequenced on a Biosystem 477A Protein Sequencer with a 120A PTH Amino Acid Analyzer equipped with a PTH, 220x2.1 mm I.D., 5μ column (Applied Biosystem).
A mixture of 5 peptides, at 2 mg/mL of each peptide, were mixed at an equal ratio. The peptide mix and the isolated bioactive peak (GH47-4) were rechromatographed using a semi-preparative Delta Pak C18-RP, 300x7.8 mm I.D., 5 µ, 100 Å column (Waters) at the same conditions described above. The sample was monitored by UV absorbance at 214 nm using a Model 993 Diode Array Detector (Waters).

Quantitative Purification of Root-Inhibiting Compounds from Gluten Hydrolysate

To compare the unit of activity of each subsample from the purification steps described above, 3 replicates of the complete procedure were performed in the following manner. Each of the subsamples from the purification protocol was dried using a Model 10-100 Virtis freeze dryer (Virtis Company Inc., Gardiner, NY) and dry weights were measured.

An aqueous solution of gluten hydrolysate (GH1) with a dry weight of 106.5 mg/mL was filtered through a 0.2 µm membrane filter. The filtrate (GH7) was collected and diluted in series for perennial ryegrass bioassays.

A volume of 30 mL of 95 mg/mL gluten hydrolysate filtrate (GH7) was loaded onto the gel filtration column and eluted with D.D. H2O at a flow rate of 1.6 mL/min. The effluent was collected in 80 tubes (GH17s) with 270 drops per tube. The GH17s samples were diluted with D.D. H2O at a 1:500 (v:v) ratio and absorbance at 214 nm was measured. Selected fractions were bioassayed on perennial ryegrass seeds. The #53GH17 was diluted in a dilution series and bioassayed to determine dose response. Eleven bioactive-compound-containing
tubes, from #48GH17 to #58GH17, were saved and subjected to further purification steps.

The 11 fractions were injected into individual C18-Sep Pak cartridges. The flow-through (GH27-1) was collected and bioassayed in a dilution series. Sample GH27-1 was subjected to HPLC (Waters Chromatography Division, Milford, MA) for further isolation. A 4.6 mg sample per run of GH27-1s, from #48GH27-1 to #58GH27-1, was injected onto a Delta Pak C18-RP, 300x7.8 mm I.D., 5 μ, 100 Å column (Waters). A gradient of methanol in D.D. H2O from 0 to 5% in 10 min at a flow rate of 4 mL/min was monitored at a UV absorbance of 214 nm using Waters Model 450 Variable Wavelength Detector (Waters) with 2.0 AUFS. Based on a pre-run chromatogram, the bioactive peak eluted at a retention time of 2.59 minutes, which had 21% of the total area was collected. The same peak from 20 runs were pooled, freeze dried and diluted with D.D. H2O for a bioassay dose response curve.

Validation of the Inhibitory Activity of the Pure Compounds

To confirm the root-inhibiting activity of the compounds identified by the ISU Protein Facility as described in phase II, five synthetic pure dipeptides, Glutaminyl-glutamine (Gln-Gln) (BACHEM Bioscience, Inc., Philadelphia, PA), alaninyl-asparagine (Ala-Asn), alaninyl-glutamine (Ala-Gln), glycinyl-alanine (Gly-Ala), and alaninyl-alanine (Ala-Ala) (Sigma) were obtained commercially and subjected to petri dish bioassay. The Ala-Gln and Gly-Ala were also bioassayed on soil.

In the petri dish bioassay, Gln-Gln, Ala-Asn, Ala-Gln, Gly-Ala, and Ala-Ala were prepared in aqueous solution in a concentration series of 0, 0.3, 0.5,
0.8, 1.0, 1.2, 1.5, and 2.0 mg/mL. The unit of activity of each dipeptide was determined. The Gly-Ala was replicated 3 times, and all the others were replicated 2 times.

Two mixtures of 2 mg/mL aqueous solution of Gln-Gln, Ala-Asn, Ala-Gln, Gly-Ala, and Ala-Ala were also bioassayed. Mixture #1 had the ratio of 2:11:1.5:3:9 (by volume) of these dipeptides based on the ratio of the peak height on the chromatogram from the Protein Facility. Mixture #2 had equal volumes of each dipeptide.

In the second study designed to verify the bioactivity of the dipeptides on soil, Ala-Gln and Gly-Ala were applied to 56.3 cm² plastic pots filled with the same type of soil as described in study 1. The test species was creeping bentgrass. The 2 dipeptides were applied at the rates of 0, 88.8, 177.6, 355.2, 710.4, 1065.7, 1776.2, and 3552.4 mg/dm². All pots were placed in growth chamber that was maintained at the conditions described in the second phase of study. Data were collected 21 days after treatment on percentage survival of seedlings and on the mean length of roots.

Eight other dipeptides and 6 amino acids were tested for their root-inhibiting activity on perennial ryegrass seeds. The eight dipeptides were alaninyl-glutamic acid (Ala-Glu), alaninyl-glycine (Ala-Gly), glycyl-asparagine (Gly-Asn), glycyl-glutamine (Gly-Gln), glycyl-glutamic acid (Gly-Glu), serinyl-asparagine (Ser-Asn), serinyl-glutamine (Ser-Gln), and serinyl-glutamic acid (Ser-Glu) (Sigma, MO), and the 6 amino acids were L-alanine (Ala), L-asparagine (Asn), L-aspartic acid (Asp), glycine (Gly), L-glutamine (Gln), and L-glutamic acid (Glu) (Sigma Chemical Co., St. Louis, MO). A solution of each dipeptide was prepared in D.D. H₂O at a
concentration of 2 mg/mL and tested for its bioactivity using the perennial ryegrass petri dish bioassay method. Five dilutions at 0, 0.5, 1.0, 1.5, and 2.0 mg/mL of the 6 amino acids, Ala, Asn, Asp, Gly, Glu, and Glu, and of 2 dipeptides, Gly-Glu, and Ala-Gly, were prepared and bioassayed using the same method.

Smooth crabgrass was also used to determine the activity of the root-inhibiting compounds. Four concentrations of GH1, mixture #2 of the 5 identified dipeptides, Ala-Gln, Ala-Gly, Ala, and Gly at 0, 1, 1.5, and 2.0 mg/mL in D. D. H2O were prepared. Petri dish bioassays were set up in the same manner as previously established, except 10 smooth crabgrass seeds were placed on the moistened Whatman #1 filter paper.

Statistical Analysis

An analysis of variance (ANOVA) was conducted using StatView (Abacus Concepts, 1991). Means were separated by the protected least significant difference (LSD) using Fisher's test (Snedecor and Cochran, 1989).
RESULTS AND DISCUSSION

Selection of a Water Soluble Sample fromCorn Gluten Meal

Study 1

All samples, except the alcohol insoluble fraction of gluten filtrate (#8), reduced crabgrass establishment compared to the control at the 4 test rates (Figure 1). The proteinase hydrolysates of corn gluten meal (#2 and 4), and the proteinase hydrolysates of corn gluten liquid (#5 and 10), were more effective root inhibitors than other test samples.

The bacterial proteinase hydrolyzed gluten sample (#5), which reduced the establishment of crabgrass seedlings by 76%, 96%, 100% and 100% at the 0.86, 1.72, 3.44, and 6.88 g/dm\(^2\) rates, respectively, was the most active of the gluten hydrolysates. The fungal proteinase hydrolysate (#3) was the least effective of the hydrolysate samples.

At 35-DAS, all 12 samples had a stimulatory effect on crabgrass growth at rates of 0.86 and 1.72 g/dm\(^2\) (Figure 2). The stimulation was likely due to a nitrogen (N) response. At rates of 3.44 and 6.88 g/dm\(^2\), several treatments reduced fresh clipping weight, with the bacterial proteinase hydrolyzed gluten sample (#5) being the most effective.

In the petri dish test, four proteinase hydrolyate samples (#2, 4, 5, and 10) and the gluten filtrate treated with a cation resin (#9) inhibited the germination of both grass species (Figure 3 and Figure 4). The fungal proteinase hydrolysate (#3) inhibited germination at the 0.355g/dm\(^2\) rate, only.
Figure 1. The effect of 12 corn gluten meal derived materials on crabgrass establishment on soil in the greenhouse. A seeding rate of 0.11 g was used on 58 cm² pots. Data are the number of living crabgrass plants at 18 days-after-seeding (DAS). Error bars indicate standard error of the mean (SE) for each treatment. The controls averaged 95 ± 12 (n=3) plants per pot.
Figure 2. The effect of 12 corn gluten meal derived materials on the fresh clipping weight of crabgrass grown on soil in the greenhouse collected at 35 DAS. A seeding rate of 0.11 g was used on 58 cm² pots. Error bars indicate SE for each treatment. The controls averaged 1.240 ± 0.535 g (n=3) per pot.
Figure 3. The effect of corn gluten meal derived materials on germination of crabgrass bioassayed in the growth chamber. Eighteen seeds were placed on a 42.25 cm² blotter paper soaked with 7 mL test solution in a petri dish which was incubated in chamber with fluorescent light for 16h and dark for 8h at 25°C for 21 days. The control had 67 ± 5% (n= 4) germination.
Figure 4. The effect of corn gluten meal derived materials on germination of creeping bentgrass tested in the growth chamber. Bioassay conditions were the same as in Figure 3. The control had 61 ± 9% (n= 4) germination.
Corn gluten meal (#1) was less active than any other test sample for the inhibition of both species.

The corn gluten hydrolysate (#5) completely stopped germination of creeping bentgrass at application levels above 0.118 g/dm$^2$, and completely stopped germination of crabgrass at application levels above 0.236 g/dm$^2$. The petri dish bioassay required lower levels of sample than the soil bioassay to inhibit germination. The definition of germination in the petri dish study included any seed with a protruding radicle or shoot. This may have been misleading because many of the seeds that were counted as germinated seed had greatly reduced root growth. In the later studies, germination was redefined to include any radicle measuring 0.5 cm or longer.

Study 2

In the greenhouse study, plant response was based on the average plant height and fresh clipping weight of creeping bentgrass plants at 28 DAS (Figure 5). All samples stimulated growth at rates of 0.78 and 1.56 g/dm$^2$ due to the nitrogen release from the materials. The bacterial proteinase hydrolysate of corn gluten solution (#15), which was equivalent to #5 in study 1, was the most effective inhibitor in this study. The corn steep liquor samples, #17 and 18, were ineffective and stimulated growth at most rates.

Perennial ryegrass was also used as a bioassay species, because of its rapid germination. The test samples were generally less effective on the ryegrass than on the bentgrass (Figure 6). The bacterial proteinase hydrolysate sample (#15) started showing inhibition at rates higher than 4.69 g/dm$^2$, and was the most effective of the materials tested.
Figure 5. The effect of corn gluten meal derived materials on bentgrass establishment bioassayed on soil in the greenhouse at 28 DAS. A seeding rate of 0.1 g was used on 64-cm² pots. The control had 3.0 ± 0.8 cm and 0.85 ± 0.13 g (n=5) for average plant height and fresh clipping weight, respectively.
Figure 6. The effect of corn gluten derived materials on the fresh clipping weight of perennial ryegrass bioassayed on soil in the greenhouse. Data were collected at 27 DAS. A seeding rate of 1.0 g was used on 64 cm² pots. The control had 0.78 ± 0.07 g (n=4).
In the petri dish study, germinated seeds were defined as those with a radicle longer than 0.5 cm extruded from the seed coat. The gluten hydrolysate sample (#15) was the most active among all 6 test samples in this study (Figure 7). It inhibited germination at all test rates, and stopped germination at rates of 0.056 g/dm² and higher. The total ion exchange gluten hydrolysate sample (#16) had slightly less activity than the gluten hydrolysate. Two gluten meal samples (#13 and 14) were less active than gluten hydrolysate. The soluble corn steep liquor sample (#17) had no inhibitory effect at all test rates, while the insoluble corn steep liquor (#18) inhibited germination at rates higher than 0.139 g/dm².

Study 3

In study 3, samples derived from corn gluten, wheat gluten, and soybean, were bioassayed using perennial ryegrass. All test samples had higher plant heights and more fresh clipping weight than the control at 28 DAS at the rates of 0.78 and 1.56 g/dm² (Figure 8). Corn gluten hydrolysate (#24) and soybean meal (#25) started to show inhibition of plant growth at the rate of 3.13 g/dm². Wheat gluten (#19) and its hydrolysate (#20) were less active and required 9.38 g/dm² to achieve similar effects.

Sample #13, tested in study 2, was used as a reference. It is gluten meal mixed with corn hulls and germs with a 60% protein content. This sample as well as the gluten filter cake sample (#23) did not show any inhibitory effect.

In the petri dish study, the wheat gluten and its hydrolysate showed inhibition at all test rates and completely inhibited radicle emergence at 0.139 g/dm² (Figure 9). Two granulated gluten filter cake samples (#21 and #22) had
Figure 7. The effect of corn gluten meal derived materials on the germination of perennial ryegrass tested in the growth chamber. Sixteen seeds were placed on 2 layers of 36-cm² blotter paper moistened with 6 mL test solution in a petri dish. All dishes were incubated in a chamber equipped with fluorescent light at 25°C for 16h and dark at 15°C for 8h at 25°C for 14 days. Geminated seed was defined as seed with radicle longer than 0.5 cm. The control had 94 ± 5% germination.
Figure 8. The effect of grain derived materials on perennial ryegrass establishment tested on soil in the greenhouse. A seeding rate of 0.5 g was used on 64-cm² pots. Data were collected at 28 DAS. The control had 7.7 ± 0.5 cm and 1.78 ± 0.44 g (n=7) for average plant height and fresh clipping weight, respectively.
Figure 9. The effect of crop derived materials on the germination of perennial ryegrass in the growth chamber. The bioassay conditions were the same as in Figure 8. The control had 96% (n=6) germination.
less activity compared to wheat gluten and its hydrolysate (#19 and #20). The wheat gluten hydrolysate had higher inhibitory activity than wheat gluten itself. It could completely inhibit germination at rates of 0.139 g/dm² and higher. The soybean meal and corn gluten hydrolysate were not tested in the growth chamber because of the shortage of sample at the time of testing.

Study 4

Based on the results from the previous studies, bacterial proteinase hydrolysate was found to be the most active sample. On soil in the greenhouse, gluten hydrolysate (#26) and its fractionated samples (#27, 28, 29, and 30) were more active than gluten meal (#14) and granulated gluten filter cake samples (#21 and 22) (Figure 10). Complete inhibition was observed at rates higher than 3.13 g/dm², except for the gluten hydrolysate precipitate (#27) which is a white solid and insoluble in water. There were at least 2 groups of compounds that resulted in root inhibition. One was water soluble and passed through the filtration system, whereas the other was water insoluble. The water soluble fractions of the gluten hydrolysate (#28, 29, and 30) were found to be more active than the water insoluble fraction (#27).

In the petri dish study, corn gluten hydrolysate related samples were more active than corn gluten meal, #14 (Figure 11). The soluble fractions of corn gluten hydrolysate (#28, 29, and 30) were more active than the insoluble fraction (#27). The most active sample was the original corn gluten hydrolysate (#26).

In bioassays used to derive a dose response of samples #26, 28, 29, and 30, it was demonstrated that roots were inhibited more by the corn gluten
Figure 10. The effect of corn gluten meal derived materials on the fresh clipping weight of perennial ryegrass grown on soil in the greenhouse collected on 31 DAS. A seeding rate of 1.0 g was used on 64-cm² pots. The control had 1.43 ± 0.60 g (n= 8).
Figure 11. The effect of corn gluten meal derived materials on the germination of perennial ryegrass tested in the growth chamber. The bioassay conditions were the same as in Figure 7. The control had 96% (n=6) germination.
hydrolysate and its water soluble fractions than were shoots (Figure 12). It indicates that the response of roots is more sensitive than that of shoots.

It was also determined that the measurements of root and shoot lengths of perennial ryegrass seedlings offer more information on plant response than germination and can provide a better activity index. Root length was determined to be the most useful as an index in the study of the herbicidal activity of the corn gluten meal related samples.

It was demonstrated in all four studies, that the corn gluten hydrolysates were a more effective inhibitor than the corn gluten meal. In soil bioassays, treatments with rates above 4.69 g/dm$^2$ of the gluten hydrolysates resulted in complete inhibition of plant establishment. Rates of 6.88 g/dm$^2$ were required for the corn gluten meal to achieve complete inhibition. The petri dish bioassay is a quicker and more economical method to monitor the inhibitory activity than is the soil bioassay, and root length reduction of seedlings was determined to be the best measurement of herbicidal activity.

These corn gluten hydrolysates are a proteinase hydrolyzed product of corn gluten meal. They are formed by a process comprised of treating the corn gluten slurry with amylases, and then treating it with one or more proteinases, followed by the removal of the water. The proteinase used to hydrolyze corn gluten meal did not contribute to the inhibition of the germination of perennial ryegrass (data not shown).

The bacterial proteinase gluten hydrolysate was the most highly bioactive, water-soluble material, which made it a suitable choice for the isolation and identification process.
Figure 12. Dose response curves of fractionated corn gluten hydrolysate samples on (A) root length and (B) shoot length of germinating perennial ryegrass seeds tested in the growth chamber. The control had a root length of 48 ± 5 mm and a shoot length of 39 ± 3 mm (n=7). The LSD(0.05) used to separate means for root length and shoot length were 6.2% and 11.5%, respectively.
Isolation and Identification of Bioactive Compound(s) from Corn Gluten Hydrolysate

During experiments designed to select the water soluble samples for isolation and identification of the bioactive compound(s), the bacterial proteinase gluten hydrolysate was found to be the most suitable. A 10% aqueous solution of the corn gluten hydrolysate (GH1) was filtered through a 0.2 µm membrane filter and the gluten hydrolysate filtrate (GH7) was collected. In a perennial ryegrass bioassay of the GH7, root growth was stopped at approximately 2 mg/mL and shoot growth at 11 mg/mL (Figure 13).

One 'unit of activity' was defined as the amount of material required to reduce root length compared to the control by 50% under the conditions of the bioassay developed in this study. The GH7 reduced rooting by 50% at approximately 1.4 mg/mL (Figure 13).

The Sephadex G-15 gel filtration was calibrated with blue dextran and NaCl. Tubes in the range of #31 to 56 were found to be within the elution volume of the column (Figure 14). Thirty mL of the GH7 was loaded on to the column and 80 fractions (GH17s) were collected. Perennial ryegrass bioassays were conducted on the samples in selected tubes. The samples in tubes #50 to #56 (#50GH17 to #56GH17) completely inhibited both root and shoot growth (Figure 14). Materials in tubes #43 to 49 and 57 to 65, completely inhibited roots but shoots were reduced less than 50%. This indicates that there may be more than one bioactive compound in the corn gluten hydrolysate aqueous solution, or that the root systems of perennial ryegrass are inhibited at lower concentrations of a single bioactive compound than are the shoots.
Figure 13. The effect of gluten hydrolysate aqueous filtrate (GH7) on seedling growth of germinating perennial ryegrass seeds tested in the growth chamber. Ten seeds were placed on 1 layer of 7 cm diameter Whatman #1 filter paper containing 1 mL test solution in each petri dish. All dishes were incubated for 10 days in the growth chamber with 70 umol/sec/m² fluorescent lighting at 25°C for 16h, 15°C dark for 8h. The control had a root length of 45 ± 7 mm and a shoot length of 36 ± 4 mm (n=7). The LSD(0.05) were 13.4 and 17.5%, respectively. Error bars indicate 1 standard deviation for each treatment.
Figure 14. Calibration of the Sephadex G-15 column and bioassay results for selective fractions of GH17s. Blue dextran and NaCl dissolved in D.D. H₂O were loaded onto a 28x998mm (630mL) Sephadex G-15 column and eluted with D.D. H₂O at a flow rate of 1.6 mL/min. Eighty fractions with 270 drops of effluent per fraction were collected and conductivity was measured. Thirty mL of 10% gluten hydrolysate filtrate (GH7) was loaded onto column, eluted and collected in the same way. Selective fractions were bioassayed with the perennial ryegrass. Bioassay conditions were the same as in Figure 13. The control had a root length of 42 ± 10 mm and a shoot length of 34 ± 5 mm (n=7). The LSD(0.05) were 13.4% and 12.4% for root length and shoot length, respectively.
Tube #53 collected from the gel filtration column (#53GH17s) was subjected to 2 different purification steps. A fractionation method on HPLC using semi-preparative C18-RP column is shown in Figure 15. Seven fractions were collected and bioassayed. Four fractions showed root-inhibiting activity, two of them showed no absorbance at UV 280 nm (Figures 15 and 16). Fractions #1 and #3 reduced root length by more than 95%. Fraction #1, that was eluted in the first 10 min on the C18-RP column, is more polar than fraction #3. This fraction was selected for isolation and identification of the active root-inhibiting-compound(s).

A C18 Sep-Pak cartridge cleanup procedure was used prior to HPLC separation. The first fraction was retained in GH27-1 after passing #53GH17 through C18 Sep-Pak cartridge (Figure 17). The GH27-1 was then loaded on to a semi-preparative C18-RP HPLC column (Rainin), and monitored at UV 214 nm (Figure 18). Twelve fractions were collected from the HPLC effluent. This step was repeated six times and each of the 12 fractions were pooled to have enough material for the bioassay. Fraction #4 (GH47-4), which corresponded to the second major peak (Figure 18), was the most inhibitory, followed by fraction #5 (Figure 19).

The GH47-4 was subjected to amino acid analysis (Table 4). The bioactive peak was shown to have 15 to 17 peaks on capillary electrophoresis (data not shown) and it was suspected to contain more than one peptide. The GH47-4 was then derivatized with phenylisothiocyanate (PITC) to form phenylthiocarbamyl (PTC) derivatives that are more hydrophobic than their un-derivatized counterparts. The derivatized GH47-4 was resolved from a narrow-bore C18 column on HPLC into 7 peaks that were collected and
Figure 15. Elution profile and fractionation of #53GH17 on reversed-phase C18 HPLC column.
Column: Dynamax C18-RP 250x10.0 mm I.D. (Rainin). HPLC instrument: ISCO coupled to a UV
detection system. Conditions: linear AB gradient from 0% to 25% B in 50 min, 25% to 100% B in 25
min, 100% B for 15 min, and 100% to 0% B in 10 min, where eluent A is D.D. H2O and B is 100%
MeOH; flow-rate 4.0 mL/min. Detection: UV at 280 nm, 0.02 AUFS. Sample= 12 mg of #53GH17.
Figure 16. Bioactivity of fractions of gluten hydrolysate collected from HPLC effluent tested by perennial ryegrass bioassay in the growth chamber. Fractionation conditions are given in Figure 17. The bioassay conditions were the same as in Figure 13. The control had a root length of $48.7 \pm 9.9$ mm and a shoot length of $45.9 \pm 6.4$ mm ($n=7$). The LSD (0.05) were 13.5% and 15.1% for root length and shoot length, respectively. Error bars indicate 1 standard deviation for each treatment.
Figure 17. Elution profiles of #53GH17 collected from Sephadex G-15 on reversed-phase C18 HPLC column (A) without and (B) with passing through Sep Pak cartridge. Column: Hibar C18-RP 250x4.6 mm I.D (EM Separation). Condition: linear AB gradient the same as Figure 17, where eluent A was D.D. H₂O and B was 100% MeOH; flow rate: 1 mL/min. Detection: UV at 280 nm. Sample: (A) 5 mg of #53GH17 and (B) 0.08 mg of #53GH27-1.
Figure 18. Elution profile and fractionation of GH27-1, flow-through from a C18 Sep-Pak cartridge, on reversed-phase C18 HPLC column. Column: Dynamax C18-RP 250x10.0 mm I.D. (Rainin). Conditions: linear AB gradient from 0% to 5% B in 10 min, 5% to 100% B in 2 min, 100% B for 7 min and 100% to 0% B in 1 min, where eluent A is D.D. H₂O and B is 100% MeOH; flow-rate of 4.0 mL/min. Detection: UV at 214 nm, 1.0 AUFS. Sample: 6 mg of #53GH27-1.
Figure 19. Bioactivity of fractions of GH47-4 collected from the HPLC effluent (GH47) using the perennial ryegrass bioassay in the growth chamber. Data were generated from the combination of 6 HPLC runs on Dynamax C18-RP 250x10.0 mm I.D. column; fractionation method is described in Figure 18. Bioassay conditions were the same as in Figure 13. The control had root length of 51 ± 12 mm and shoot length of 37 ± 8 mm (n=7). The LSD(0.05) were 14% and 16.5% for root length and shoot length, respectively. Error bars indicate 1 standard deviation for each treatment.
Table 4. Amino acid analysis of a root-inhibiting peak (GH47-4) isolated from corn gluten hydrolysate

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>pmol (Internal Standard Corrected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>217</td>
</tr>
<tr>
<td>Aspartic acid + Asparagine</td>
<td>55</td>
</tr>
<tr>
<td>Glutamic acid + Glutamine</td>
<td>1632</td>
</tr>
<tr>
<td>Glycine</td>
<td>188</td>
</tr>
<tr>
<td>Serine</td>
<td>391</td>
</tr>
<tr>
<td>Threonine</td>
<td>261</td>
</tr>
<tr>
<td>Valine</td>
<td>37</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>31</td>
</tr>
<tr>
<td>Leucine</td>
<td>29</td>
</tr>
</tbody>
</table>
sequenced by a modified sequencing method (Figure 20). Each peak was sequenced for 5 cycles. The first five peaks were identified as the following dipeptides: Gln-Gln, Ala-Asn, Ala-Gln, Gly-Ala and Ala-Ala.

Samples of GH47-4 and a mixture of the 5 peptides were rechromatographed on a new Delta-Pak C18-RP 300x7.8 mm I.D., 5 μm, 100Å column (Waters). The HPLC instrument was coupled to a photodiode array detector with scanning capacity (Figure 21). Both samples had the same retention time and their spectrums had strong UV absorbance between 190 and 220 nm.

The purification procedures developed in this phase were able to isolate root-inhibiting compounds from corn gluten hydrolysate aqueous solution. A summary of the isolation procedure is shown in Figure 22.

**Quantitative Purification of Root-Inhibiting Compounds from Corn Gluten Hydrolysate**

An aqueous solution of gluten hydrolysate (GH1) containing 106.5 mg dry weight per mL of solution was prepared. A dose response curve of GH1 demonstrated that 1.5 mg of this material provided 1 unit of activity (Figure 23). The dry weight of the aqueous filtrate (GH7) was 95 mg per mL. The GH7 provided 1 unit of activity at 1.5 mg (Figure 24).

Thirty mL of GH7, with a total of 2850 mg material, was loaded onto a Sephadex G-15 gel filtration column. The dry weight of the 80 tubes collected from the column was correlated with UV absorbance at 214 nm (Figure 25). The active fractions from tubes #48 to 58 inhibited root growth 100% and shoot growth at least 50%. The amount of material in the tubes (#48GH17 to
PEAK I.D.

1. Gln-Gln
2. Ala-Asn
3. Ala-Gln
4. Gly-Ala
5. Ala-Ala

Figure 20. Elution profile and identification of PTC-derived GH47-4 on narrow-bore C18-RP HPLC column. Column: Vydac 250x2.1 mm I.D. Conditions: linear AB gradient of 5% to 45% in 35 min, where eluent A is 0.1% trifluoroacetic acid (TFA) in water and eluent B is 0.08% TFA in acetonitrile; flow-rate at 300 ul/min. Detection: UV at 214 nm. The five peaks were identified by peptide sequencing as Gln-Gln, Ala-Asn, Ala-Gln, Gly-Gln, and Ala-Ala.
Figure 21. Elution profile of GH47-4 and a 5-dipeptide mixture on reversed-phase C18 HPLC column. Column: Delta-Pak C18-RP 300x7.8 mm I.D. (Waters). HPLC instrument: Beckman system coupled to Photodiode Array Detector (Waters). Conditions: linear AB gradient from 0% to 5% B in 10 min, where eluent A was D.D. H₂O and B was 100% MeOH; flow-rate of 4.0 mL/min. Detection: UV at 214 nm, 1.0 AUFS. Sample: (A) GH47-4 (B) 5-dipeptide mixture.
Gluten Hydrolysate

\[ \rightarrow \text{D.D. } H_2O \]

Aqueous Solution (GH1)

\[ \rightarrow 0.2 \text{ um Membrane Filter} \]

Filtrate (GH7)

\[ \rightarrow \text{Gel Filtration, Sephadex G-15} \]

Bioactive Fraction (GH17s)

\[ \rightarrow \text{C18 Sep-Pak Cartridge} \]

\[ \rightarrow \text{10% MeOH} \]

\[ \rightarrow \text{100% MeOH} \]

Flow Through (GH27-1)

\[ \rightarrow \text{HPLC C18-RP Prep Column} \]

\[ \rightarrow \text{MeOH in } H_2O \text{ Linear Gradient} \]

Bioactive Peak (GH47-ACPK)

Figure 22. Purification protocol for isolating root-inhibiting compounds from corn gluten hydrolysate.
Figure 23. Dose response curve of GH1, the gluten hydrolysate aqueous solution, on root growth of germinating perennial ryegrass seeds tested in the growth chamber. The bioassay conditions were the same as in Figure 13. The control had a root length of 46 ± 7 mm (n=7). The LSD (0.05) was 13.0%. Error bars indicate 1 standard deviation for each treatment.
Figure 24. Dose response curve of GH7, gluten hydrolysate aqueous solution filtrate. Perennial ryegrass bioassay conditions were the same as in Figure 13. The control had a root length of 46 \pm 8\;\text{mm}\; (n=7). The LSD (0.05) was 9.5\%. Error bars indicate 1 standard deviation for each treatment.
Figure 25. Quantity and UV absorbance at 214 nm of GH17s, collected from Sephadex G-15 column. Total of 2580 mg gluten hydrolysate filtrate (GH7) was loaded onto a Sephadex G-15 gel filtration column, eluted with D.D. H₂O at a flow rate of 1.6 mL/min. Eighty fractions of 270 drops of effluent per fraction were collected. The absorbance of a 1:500 ratio dilution was measured at 214 nm.
#58GH17) ranged from 100.3 to 146.3 mg dry weight per tube (Figure 26). A
dose response was conducted on #53GH17. Less than 1.1 mg were required to
achieve 50% reduction of root length (Figure 27).

The bioactive GH17s samples were passed through separate C18 Sep-
Pak cartridges. The flow through (GH27-1s), that had 53.5% yield on a dry
weight basis of GH17s, was collected. A dose response curve of #53GH27-1
showed that approximately 1.0 mg was needed for 1 unit of activity (Figure 28).

The GH27-1 was loaded on to a Delta-Pak C18-RP (Waters) column and
eluted with a 0 to 5% methanol in water linear gradient (Figure 29). The
bioactive peak (GH47-ACP) that was eluted at a retention time of 2.59 min
accounted for 21% of total area in the chromatogram. The pooled GH47-ACP
from twenty HPLC runs was dried and bioassayed. Approximately 1.1 mg of
GH47-ACP were required for 1 unit of activity (Figure 30).

The purification progress expressed as the amount of material required
for 50% root inhibition compared to the control for each subsample is shown in
Figure 31. The units of activity were significantly different between
subsamples GH1 and GH17, GH1 and GH27-1, and GH1 and GH47, but not in
other comparisons (Table 5). Each subsample was replicated three times
except GH1 which had four replicates. The dose response curves of replicates
for each subsamples are in appendix A. The quantitative purification summary
following the described protocol is given in Table 5.

**Validation of the Inhibitory Activity of the Pure Compounds**

The root inhibitory activity of the five identified dipeptides was
confirmed by the perennial ryegrass bioassay (Figure 32). The most bioactive
Figure 26. Quantity and bioassay results of selected tubes collected from Sephadex G-15 gel filtration column. A total of 2580 mg gluten hydrolysate filtrate (GH7) was loaded onto Sephadex G-15 gel filtration column, eluted with D.D. H₂O at flow rate of 1.6 mL/min. Eighty fractions of 270 drops of effluent per fraction were collected and selectively bioassayed with perennial ryegrass. Control had root length of 42 ± 10 mm and shoot length of 34 ± 5 mm (n=7). The LSD (0.05) were 12.4% and 12.6%, respectively.
Figure 27. Dose response curve of #53 GH17, an active fraction collected from gel filtration. The conditions of the perennial ryegrass bioassay were the same as in Figure 13. The control had a root length of $47 \pm 6$ mm ($n=7$). The LSD(0.05) was 9.2%. Error bars indicate 1 standard deviation for each treatment.
Figure 28. Dose response curve of GH27-1, the flow through collected from a C18 Sep-Pak cartridge. The conditions of the perennial ryegrass bioassay were the same as in the Figure 13. The control had a root length of 47 ± 6 mm, (n=7). The LSD(0.05) was 10.7%. Error bars indicate 1 standard deviation for each treatment.
Figure 29. Elution profile of GH27-1 and GH47-ACPK that was eluted at 2.59 min on reversed-phase C18 HPLC column. Column: Delta-Pak C18-RP 300x7.8 mm I.D. (Waters). HPLC instrument: Waters tri-module system coupled to a variable wavelength detector. Conditions: linear AB gradient from 0% to 5% B in 10 min, where eluent A was D.D. H₂O and B was 100% MeOH, flow-rate of 4.0 mL/min. Detection: UV at 214 nm, 1.0 AUFS. Sample: 6 mg of #53GH27-1.
Figure 30. Dose response curve of GH47-ACP, the active peak collected from HPLC. The conditions of the perennial ryegrass bioassay were the same as in Figure 13. The control root length was 51 ± 8 mm, (n=7). The LSD(0.05) was 11.5 %. Error bars indicate 1 standard deviation for each treatment.
Figure 31. Comparison of root-inhibiting activity among subsamples of corn gluten hydrolysate expressed as the amount of material required to inhibit 50% of the control root length. Error bars represent the standard error of the mean; each subsample had 3 replicates except GH1 which had 4 replicates. The LSD(0.05) was 0.45. Dose response curves of each subsamples are given in 'APPENDIX A'.
Table 5. Quantitative purification of root-inhibiting compounds from corn gluten hydrolysate

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Unit of Activity(^a)</th>
<th>Specific Activity</th>
<th>Fold Purification</th>
<th>Total Activity</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Subsample)</td>
<td>(mg/mL ± S.E.(^b))</td>
<td>(Units/mg)</td>
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<td></td>
<td></td>
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<tr>
<td>Crude Extract (GH1)</td>
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<td>1.0</td>
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<td>100</td>
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<tr>
<td>Membrane Filter (GH7)</td>
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<td>0.8</td>
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<tr>
<td>Gel Filtration (GH17)</td>
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<tr>
<td>Solid Phase Extraction (GH27-1)</td>
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<td>0.9</td>
<td>1.5</td>
<td>671</td>
<td>31</td>
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<tr>
<td>C18-RP HPLC (GH47-ACPK)</td>
<td>1.1 ± 0.1</td>
<td>0.9</td>
<td>1.5</td>
<td>66</td>
<td>3</td>
</tr>
</tbody>
</table>

\(^a\)Dose response curves of each subsample are given in ‘APPENDIX A’.

\(^b\)S.E. is the standard error of the mean for each subsample. LSD(0.05)= 0.5.
Test Peptide Sample

Figure 32. Comparison of root-inhibiting activity among 5 dipeptides and 2 mixtures of these 5 peptides expressed as the amount of material required per unit of activity. Mixture #1 had the five peptides in a ratio of 9:2:11:1.5:3. This was based on the proportion of peak height in Figure 20. Mixture #2 had an equal ratio of the 5 peptides. All test samples had 2 replicates except Mixture #1 which had 1 and Gly-Ala which had 3 replicates. Error bars indicate SE for each sample. The LSD(0.05) was 0.32. The dose response curves of each test peptide are given in 'APPENDIX B'.
dipeptides were Ala-Ala and Gly-Ala. The Ala-Gln, Ala-Asn, and Gln-Gln were less active. The unit activity of Ala-Asn and Gln-Gln was significantly higher (p< 0.05) than that of Gly-Ala and Ala-Ala.

Two mixtures of the 5 dipeptides were tested against the individual dipeptides. Mixture #1 was prepared according to the ratio of peak heights of 5 dipeptides in GH47-4 (Figure 22). The ratio was 9: 2: 11: 1.5: 3 for Gln-Gln, Ala-Asn, Ala-Gln, Gly-Ala, and Ala-Ala, respectively. Its unit of activity was 0.8 mg (Figure 32). Mixture #2 that had an equal ratio of the 5 dipeptides, required 0.6 mg to achieve 1 unit of activity. This is due to higher proportion of the more active dipeptides, Gly-Ala and Ala-Ala in mixture #2. Neither mixture increased the inhibitory activity compared to the individual dipeptides, indicating no synergistic effects among these compounds. The dose response curve of each of the 5 dipeptides and the mixtures are shown in appendix B.

The comparison of root-inhibiting activity of the corn gluten hydrolysate purification subsamples, the 5 dipeptides, and their mixtures expressed on a unit of activity basis is shown in Figure 33. The crude extract, GH1, required 1.63 ± 0.21 mg to achieve 1 unit of activity. This was significantly higher than that of any other sample. The unit activity of the active peak isolated from C18-RP HPLC (GH47-ACPK) was 1.05 ± 0.08 mg which was significantly higher than that of Gly-Ala and Ala-Ala.

A soil bioassay of Ala-Gln and Gly-Ala was conducted using creeping bentgrass to verify their bioactivity on soil at the rates of from 0 to 3552 mg/dm² (Table 6). The Ala-Gln and Gly-Ala reduced the establishment of creeping bentgrass at least 50% at rates of 1776 g/dm² and above. Rooting of
Test Sample

Figure 33. Comparison of root-inhibiting activity among corn gluten hydrolysate subsamples, the 5 identified dipeptides, and 2 mixtures of these 5 dipeptides expressed as the amount for 1 unit of activity tested on perennial ryegrass. Mixture #1 had five dipeptides ratio of 9:2:11:1.5:3 based on the proportion of peak height in Figure 20. Mixture 2 had equal ratio of the 5 dipeptides. Error bars indicate SE for each sample. The LSD(0.05) was 0.39.
Table 6. Bioactivity of Ala-Gln and Gly-Ala on creeping bentgrass tested on soil grown in the growth chamber expressed as percentage (%) of grass plants surviving 25 DAS and mean root length of seedling (mm)

<table>
<thead>
<tr>
<th>Application Rate</th>
<th>Ala-Gln (%)</th>
<th>Ala-Gln (mm)</th>
<th>Gly-Ala (%)</th>
<th>Gly-Ala (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mg/dm²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>0</td>
<td>100</td>
<td>25</td>
<td>100</td>
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<td>89</td>
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<td>3552</td>
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*Dipeptides at eight levels of 0, 0.3, 0.5, 0.8, 1.0, 1.2, 1.5, and 2.0 mg were applied to the soil surface of 56.3 cm² in plastic pots.*
the creeping bentgrass was reduced by 80% or more, and seedling survival was reduced by 70% or more at 355 mg/dm² and above.

The bioactivity of 8 other dipeptides was examined with petri dish bioassays (Figure 34). The dipeptide Ala-Gly, with a unit of activity of 0.6 mg, was found to be the most inhibitory compound to both root and shoot growth of perennial ryegrass seedlings among the dipeptides tested (Figure 35). The constituent amino acids of Ala-Gly are exactly the same as Gly-Ala, one of the 5 bioactive dipeptides identified from the corn gluten hydrolysate, but the sequence was reversed. Three other dipeptides that were found to completely inhibit root emergence at the concentration of 2 mg/mL were Gly-Asn, Ser-Asn, and Ser-Gln. The least bioactive among the peptides was Gly-Glu that could not reach more than 40% with 2 mg/mL (Figures 34 and 35).

It appears that the constituent amino acids of the dipeptides are more important than their sequence for the root-inhibiting activity. The dipeptides found to inhibit rooting were commonly composed of the following amino acids; Ala, Gly, Asn, Gln, Asp and Glu.

The root-inhibiting activity of these amino acids on perennial ryegrass was examined with petri dish bioassays (Figure 36). The amino acid Asn completely inhibited perennial ryegrass root emergence at 1.0 mg and was found to be the most inhibitory of the amino acids tested. Amino acids, Ala and Gln, were quite effective at the 1.0 mg rate. The two amino acids, Asp and Glu, were found to be much less active than their corresponding amides.

The corn gluten hydrolysate crude extract (GH1), the 5-dipeptide mixture #2, Ala-Gln, Ala-Gly, Ala, and Gly were also tested on smooth crabgrass using the 1-mL petri dish bioassay. Smooth crabgrass rooting was
Figure 34. Bioactivity of 8 dipeptides at a concentration of 2 mg/mL on perennial ryegrass seedling growth. The conditions of the perennial ryegrass bioassay were the same as Figure 13. The control had a root length of 43 ± 8 mm and a shoot length of 37 ± 3 mm (n=7). The LSD(0.05) were 14.1% and 14.2% for root length and shoot length, respectively.
Figure 35. Comparison of root-inhibiting activity of two dipeptides, Ala-Gly and Gly-Glu by perennial ryegrass bioassay. The conditions of the bioassay were the same as Figure 13. The control had a root length of $51 \pm 6$ mm ($n=14$). The LSD(0.05) were 7% and 17%, for Ala-Gly and Gly-Glu, respectively.
Figure 36. Comparison of root-inhibiting activity among 6 test amino acids and Ala-Gly on perennial ryegrass. The conditions of the bioassay were the same as in Figure 13. The control had a root length of 41 ± 4 mm and a shoot length of 48 ± 3 mm (n=20). The LSD(0.05) were 4.5%, 8.4%, 4.3%, 9.4%, 9.1%, 11.7%, and 9.5%, for Ala-Gly, Ala, Gly, Asn, Gln, Asp, and Glu, respectively.
completely stopped by 1.5 mg and higher rates of all materials tested (Table 7). This indicated that crabgrass was more sensitive to the bioactive compounds than the perennial ryegrass.

Table 7. The bioactivity of selected samples tested on smooth crabgrass expressed as the percent of control root length (%)

<table>
<thead>
<tr>
<th>Test Sample</th>
<th>Percent of Controla Root Length ± S.D. b (%)</th>
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<tbody>
<tr>
<td></td>
<td>1.0 mg/mL</td>
</tr>
<tr>
<td>GH1</td>
<td>5.1 ± 0.9</td>
</tr>
<tr>
<td>5-Dipeptides Mixture 2</td>
<td>2.4 ± 4.3</td>
</tr>
<tr>
<td>Ala-Gln</td>
<td>13.3 ± 8.0</td>
</tr>
<tr>
<td>Ala-Gly</td>
<td>2.0 ± 3.5</td>
</tr>
<tr>
<td>Ala</td>
<td>7.5 ± 6.2</td>
</tr>
<tr>
<td>Gly</td>
<td>1.7 ± 2.3</td>
</tr>
</tbody>
</table>

a The control had a root length of 42 ± 8 mm (n=7).
b S.D. is the standard deviation of the root length of the seedlings in the same petri dish.
SUMMARY AND GENERAL DISCUSSION

Public awareness and concern for environmental protection and human safety has led to the search for naturally occurring compounds that are able to inhibit growth and development of weed plants. It has been reported that corn gluten meal is useful as a natural preemergence herbicide and fertilizer material for various plant production systems. The active component(s) responsible for the herbicidal activity of corn gluten meal could potentially be used as natural herbicides. The objectives of this study were to select water-soluble corn gluten meal related materials and to isolate and identify the active compound(s) responsible for the inhibitory activity.

Three species of grass plants were used to test the inhibitory activity of corn gluten meal derived samples, to select a water soluble material to be used in the isolation process. Based on the results from greenhouse and growth chamber bioassays, it was found that the samples of hydrolyzed protein from corn gluten meal were more herbicidally active than the corn gluten itself and were highly water-soluble. Gluten hydrolysate prepared with a bacterial proteinase had the highest inhibitory activity. This material was chosen for isolation and identification of the bioactive compounds.

The response of roots was more sensitive than that of shoots to the inhibitory compounds. A new petri dish bioassay using perennial ryegrass was developed for the isolation and identification process. A unit of activity was defined as the amount of material required to inhibit 50% root length of the control in the perennial ryegrass bioassay.
An aqueous solution of the corn gluten hydrolysate was prepared as the crude extract. Five dipeptides were isolated and shown through bioassays to have root-inhibiting activity. The five dipeptides were Gln-Gln, Ala-Asn, Ala-Gln, Gly-Ala, and Ala-Ala. Synthetically produced samples of these 5 dipeptides were obtained and their inhibitory effects on roots of test grass plants were demonstrated in petri dish bioassays. The units of activity were 1.05, 0.85, 0.75, 0.50, and 0.45 mg for Gln-Gln, Ala-Asn, Ala-Gln, Gly-Ala, and Ala-Ala, respectively. The mixtures of these 5 dipeptides at different ratios showed no synergistic effect on the root-inhibiting activity. However, the bioactivity was increased as increasing the proportion of the more active dipeptides in the mixture. In a soil bioassay, Ala-Asn and Gly-Ala were demonstrated to inhibit rooting of creeping bentgrass.

Eight other dipeptides were tested for their inhibitory activity using the perennial ryegrass petri dish bioassays. The Ala-Gly was found to be the most inhibitory among the test dipeptides, and Gly-Glu the least. The constituent amino acid of the dipeptide was found to be more important than the sequence for bioactivity. It was also found that Asn was the most potent root inhibitor among 6 amino acids tested, followed by Ala, Gln, and Gly.

In an additional test, smooth crabgrass was found to be more sensitive to the root-inhibiting compounds than perennial ryegrass.

The herbicidal activity of corn gluten meal was demonstrated in earlier work. It was shown in these studies that more active fractions of corn gluten meal could be isolated and that they have the potential of being used as natural herbicides.
It was found that there are several groups of chemicals in corn gluten hydrolysate responsible for the root-inhibiting activity. Five dipeptides isolated from the aqueous filtrate of the gluten hydrolysate were shown to inhibit root formation of germinating weeds. They have the potential for use as naturally occurring growth regulating compounds and as natural preemergence herbicides.

The naturally occurring 5 dipeptides and many other bioactive dipeptides could be used in combination with non-toxic, compatible carriers, including water and clay for use in agricultural situations. They could also potentially be used to fortify the corn gluten or gluten hydrolysate to increase their effectiveness as selective, preemergence herbicides. More work is needed in the field to develop corn gluten hydrolysate and the dipeptides as herbicides.

Based on the chromatographic studies, there are likely other bioactive compounds in the hydrolysate that could potentially be used as natural herbicides. More work needs to be done to isolate the naturally occurring inhibitory compounds present in corn.


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You do not understand what love is unless you have been away from home.
If you have never cried, you do not realize the blessing that laughter is.
You do not treasure good health until you are sick.

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I would also like to thank Dr. L. A. Wilson for serving on my program of study. He reminded me that as a researcher, I should never stop learning and limit myself in only one discipline.

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Two other individuals who were away from me, however, hold the most important place in my heart to motivate me. My mother, although she is an illiterate, is a pure-hearted and wonderful lady. As a widow for 26 years, she never stopped hoping the best for her children. May she be honored by her children. The deepest appreciation is for my sister, Ms. Christiana P. Liu, for her sacrifices and endless love and for her support of the family. She gives me whole-hearted support and wants the best for me. May God bless her the best.

I believe ‘Every good and perfect gift is from above, coming down from the Father of the heavenly lights’ (James, 1:17), therefore, last but not least, my heart-felt gratitude goes to God. He opened the door to a fascinating research world, guided my every step throughout the adventurous journey, and provided me everything needed in this voyage. To Him be all the glory.
APPENDIX A: DOSE RESPONSE CURVES OF CORN GLUTEN HYDROLYSATE SUBSAMPLES
Figure 37. Dose response curve of GH1. The conditions of the perennial ryegrass bioassay were the same as in Figure 13. The control had a root length of 34 ± 7 mm (n=7). The LSD(0.05) was 13.4%. Error bars indicate 1 standard deviation for each treatment.
Figure 38. Dose response curve of GH1. The conditions of the perennial ryegrass bioassay were the same as in Figure 13. The control had a root length of 52 ± 11 mm (n=7). The LSD(0.05) was 13.0%. Error bars indicate 1 standard deviation for each treatment.
Figure 39. Dose response curve of GH1. The conditions of the perennial ryegrass bioassay were conditions were the same as in Figure 13. The control root length was 46 ± 7 mm (n=7). The LSD(0.05)= 13.0%. Error bars indicate 1 standard deviation for each treatment.
Figure 40. Dose response curve of GH1. The conditions of the perennial ryegrass bioassay were conditions were the same as in Figure 13. The control root length was 45 ± 7 mm (n=7). The LSD(0.05)= 11.7%. Error bars indicate 1 standard deviation for each treatment.
Figure 41. Dose response curve of GH7. The conditions of the perennial ryegrass bioassay were the same as in Figure 13. The control root length was 45 ± 7 mm (n=7). The LSD(0.05) was 13.4%. Error bars indicate 1 standard deviation for each treatment.
Figure 42. Dose response curve of GH7. The conditions of the perennial ryegrass bioassay were the same as in Figure 13. The control root length was 46 ± 8 mm (n=7). The LSD(0.05) was 9.5%. Error bars indicate 1 standard deviation for each treatment.
Figure 43. Dose response curve of GH7. The conditions of the perennial ryegrass bioassay were the same as in Figure 13. The control root length was $50 \pm 9$ mm ($n=7$). The LSD(0.05) was 11.1%. Error bars indicate 1 standard deviation for each treatment.
Figure 44. Dose response curve of GH17. The conditions of the perennial ryegrass bioassay were the same as in Figure 13. The control root length was 47 ± 5 mm (n=7). The LSD(0.05) was 7.9%. Error bars indicate 1 standard deviation for each treatment.
Figure 45. Dose response curve of GH17. The conditions of perennial ryegrass bioassay were the same as in Figure 13. The control root length was 50 ± 8 mm (n=7). The LSD(0.05) was 10.7%. Error bars indicate 1 standard deviation for each treatment.
Figure 46. Dose response curve of GH17. The conditions of perennial ryegrass bioassay were the same as in Figure 13. The control root length was 47 ± 6 mm (n=7). The LSD(0.05) was 9.2%. Error bars indicate 1 standard deviation for each treatment.
Figure 47. Dose response curve of GH27-1. The conditions of perennial ryegrass bioassay were the same as in Figure 13. The control root length was 48 ± 7 mm (n=7). The LSD(0.05) was 14.1%. Error bars indicate 1 standard deviation for each treatment.
Figure 48. Dose response curve of GH27-1. The conditions of perennial ryegrass bioassay were the same as in Figure 13. The control root length was 43 ± 6 mm (n=7). The LSD(0.05) was 14.2%. Error bars indicate 1 standard deviation for each treatment.
Figure 49. Dose response curve of GH27-1. The conditions of perennial ryegrass bioassay were the same as in Figure 13. The control root length was 47 ± 6 mm (n=7). The LSD(0.05) was 10.7%. Error bars indicate 1 standard deviation for each treatment.
Figure 50. Dose response curve of GH47. The conditions of perennial ryegrass bioassay were the same as in Figure 13. The control root length was 47 ± 6 mm (n=7). The LSD(0.05) was 12.3%. Error bars indicate 1 standard deviation for each treatment.
Figure 51. Dose response curve of GH47. The conditions of perennial ryegrass bioassay were the same as in Figure 13. The control root length was $51 \pm 8$ mm ($n=7$). The LSD(0.05) was 11.5%. Error bars indicated 1 standard deviation for each treatment.
Figure 52. Dose response curve of GH47. The conditions of perennial ryegrass bioassay were the same as in Figure 13. The control root length was 46 ± 8 mm (n=7). The LSD(0.05) was 9.5%. Error bars indicate 1 standard deviation for each treatment.
APPENDIX B: DOSE RESPONSE CURVES OF THE FIVE DIPEPTIDES
Figure 53. Dose response curve of Gln-Gln. The conditions of perennial ryegrass bioassay were the same as in Figure 13. The control root length was $36 \pm 9$ mm ($n=7$). The LSD(0.05) was 17.2%. Error bars indicate 1 standard deviation for each treatment.
Figure 54. Dose response curve of Gln-Gln. The conditions of perennial ryegrass bioassay were the same as in Figure 13. The control root length was 44 ± 6 mm (n=7). The LSD(0.05) was 12.2%. Error bars indicate 1 standard deviation for each treatment.
Figure 55. Dose response curve of Ala-Asn. The conditions of perennial ryegrass bioassay were the same as in Figure 13. The control root length was 38 ± 5 mm (n=7). The LSD(0.05) was 10.5%. Error bars indicate 1 standard deviation for each treatment.
Figure 56. Dose response curve of Ala-Asn. The conditions of perennial ryegrass bioassay were the same as in Figure 13. The control root length was 46 ± 6 mm (n=7). The LSD(0.05) was 10.4%. Error bars indicate 1 standard deviation for each treatment.
Figure 57. Dose response curve of Ala-Gln. The conditions of perennial ryegrass bioassay were the same as in Figure 13. The control root length was 47 ± 5 mm (n=7). The LSD(0.05) was 10.5%. Error bars indicate 1 standard deviation for each treatment.
Figure 58. Dose response curve of Ala-Gln. The conditions of perennial ryegrass bioassay were the same as in Figure 13. The control root length was 48 ± 9 mm (n=7). The LSD(0.05) was 10.8%. Error bars indicate 1 standard deviation for each treatment.
Figure 59. Dose response curve of Gly-Ala. The conditions of perennial ryegrass bioassay were the same as in Figure 13. The control root length was 47 ± 5 mm (n=7). The LSD(0.05) was 7.4%. Error bars indicate 1 standard deviation for each treatment.
Figure 60. Dose response curve of Gly-Ala. The conditions of perennial ryegrass bioassay were the same as in Figure 13. The control root length was 48 ± 9 mm (n=7). The LSD(0.05) was 12.2%. Error bars indicate 1 standard deviation for each treatment.
Figure 61. Dose response curve of Gly-Ala. The conditions of perennial ryegrass bioassay were the same as in Figure 13. The control root length was 51 ± 6 mm (n=7). The LSD(0.05) was 7.4%. Error bars indicate 1 standard deviation for each treatment.
Figure 62. Dose response curve of Ala-Ala. The conditions of perennial ryegrass bioassay were the same as in Figure 13. The control root length was 47 ± 5 mm (n=7). The LSD(0.05) was 6.0%. Error bars indicate 1 standard deviation for each treatment.
Figure 63. Dose response curve of Ala-Ala. The conditions of perennial ryegrass bioassay were the same as in Figure 13. The control root length was 46 ± 6 mm (n=7). The LSD(0.05) was 7.8%. Error bars indicate 1 standard deviation for each treatment.
Figure 64. Dose response curve of mixture #1 of the 5-dipeptides. The conditions of perennial ryegrass bioassay were the same as in Figure 13. The control root length was 44 ± 6 mm (n=7). The LSD(0.05) was 9.3%. Error bars indicate 1 standard deviation for each treatment.
Figure 65. Dose response curve of mixture #2 the 5-dipeptides. The conditions of perennial ryegrass bioassay were the same as in Figure 13. The control root length was 43 ± 6 mm (n=7). The LSD(0.05) was 11.1%. Error bars indicate 1 standard deviation for each treatment.
Figure 66. Dose response curve of mixture #2 the 5-dipeptides. The conditions of perennial ryegrass bioassay were the same as in Figure 13. The control root length was 46 ± 4 mm (n=7). The LSD(0.05) was 7.0%. Error bars indicate 1 standard deviation for each treatment.