Bioactivity of a Pentapeptide Isolated from Corn Gluten Hydrolysate on *Lolium perenne L.*

D. L. Liu and N. E. Christians*

Department of Horticulture, Iowa State University, Ames, Iowa 50011, USA

Abstract. Corn gluten hydrolysate (CGH) has been observed to inhibit root formation of germinating grass seeds and has the potential for use as a natural herbicide. Five dipeptides have been isolated from the aqueous solution of CGH and proved to have greater root-inhibiting activity than the crude extract of CGH. The objective of this study was to isolate and identify other biologically active compounds from CGH with herbicidal properties. A perennial ryegrass *(Lolium perenne* L.) Petri dish bioassay was used to test for the bioactivity. A pentapeptide, Leu-Ser-Pro-Ala-Gln, was isolated from CGH by using Sephadex G-15 gel filtration and two-step C18 reversed phase high performance liquid chromatography procedures. The compound suppressed growth of both the root and the shoot of germinating perennial ryegrass. It required 0.5 mg/mL of the pentapeptide to inhibit 50% of root length in the perennial ryegrass bioassay, and this compound is more active than any of the five dipeptides isolated previously from CGH.

Key Words. Natural products—Weed control

With widespread concern over environmental and public health issues concerning the use of pesticides, research in developing environmentally safe herbicides has resulted in the investigation of natural products for weed control (Lydon and Duke 1987, Nonnecke and Christians 1993).

Some plant compounds, which can inhibit growth and development of other plants, could be used as natural herbicides or as the starting points for chemical synthesis of biodegradable herbicides. The plant-derived compounds used as natural herbicides are considered to represent an environmentally sound approach to weed control (Lax et al. 1988, Lydon and Duke 1987).

Corn gluten meal (CGM) is a byproduct of corn *(Zea mays* L.) from the wet-milling process and has been patented as a natural preemergence herbicide (Christians 1991). It inhibits root growth of germinating seeds but does not damage plants that have formed a mature root system (Christians 1993). Because CGM contains approximately 10% nitrogen by weight, it is useful as a natural preemergence herbicide and fertilizer for various plant production systems (Christians 1993, Nonnecke and Christians 1993).

Corn gluten hydrolysate (CGH), a water-soluble material derived from the action of bacterial proteinase, was found more herbicidally active against three grass species (Liu et al. 1994) than CGM both in the soil and Petri dish bioassays. It also has a greater nitrogen content and could potentially be used to fortify the CGM to improve it as a natural weed and feed material (Christians et al. 1994, Nonnecke and Christians 1993). Its high water solubility makes it sprayable and easy to apply as a herbicide in some crop production systems. It is also useful as a source for the isolation and identification of the bioactive compounds.

Five dipeptides, Gln-Gln, Ala-Asn, Ala-Gln, Gly-Ala, and Ala-Ala, were isolated in earlier work from the aqueous solution of CGH (Liu and Christians 1994). They were demonstrated to have significantly greater root inhibition activity than the crude extract of CGH in Petri dish bioassays. Other potentially bioactive compounds were found in the aqueous solution of CGH (Liu and Christians 1994). The objective of this study was to isolate and identify other bioactive compounds from CGH.

Materials and

Perennial Ryegrass Bioassays

All bioassays used in this study were performed according to the following description unless otherwise stated. One milliliter of test solution was applied to one layer of 7-cm-diameter Whatman no. 1 filter paper measuring 38.5 cm^2, which was placed in a 9-cm-diameter Petri dish. Ten perennial ryegrass *(Lolium perenne* L.) seeds were placed on the wetted filter paper. The dish was covered, sealed with Parafilm, and incubated in a growth chamber. The radiation intensity in the chamber was 90 µmol/s/m^2 with 25/15°C day/night temperature and an 8-h photoperiod. The longest root and shoot of each seedling were measured to the nearest millimeter after 7 days. The measurements of seven seedlings in each Petri dish found to have root lengths within ± I S.D. of the mean were averaged. The seven measurements of each treatment were divided by the average root length of the control that received the deionized distilled water (D.D. H$_2$O) only. The ratio was multiplied by 100, and the value was expressed as the percentage of the control length

Extraction and Purification

Gluten hydrolysate powder was dissolved in D.D. H$_2$O to make a 10% solution. This aqueous solution of gluten hydrolysate was filtered through a 0.2-µm membrane filter (German Sciences Inc., Ann Arbor, MI) The filtrate was designated as GH-f. A dilution series of GH-f was made for the perennial ryegrass bioassay.

A 28- x 998-mm (bed volume, 630 mL) Sephadex G-15 (Sigma Chemical Company, St. Louis, MO) gel filtration column was calibrated with blue dextran and NaCI aqueous solution and eluted with D.D. H$_2$O at a flow rate of 1.6 mL/min. Eighty fractions with 270 drops of effluent per fraction were collected by using a Gilson model 201 fraction collector (Gilson Medical Electronics, Inc., Middleton, WI). Conductivity of effluent of each tube was measured by using a YSI conductivity bridge (YSI, Orangeburg, NY). Thirty milliliters of GH-f was loaded onto the column, which was eluted with D.D. H$_2$O at a flow rate of 16 mL/min. The effluent (GH-g15-e) was collected in 80 tubes with 270 drops in each tube as already described. Selected tubes were bioassayed for their bioactivity by using the perennial ryegrass bioassay. Tubes 50-56 (50GH-g15-e to 56GH-g15-e) were found to contain the root- and shoot-inhibiting compound(s) as shown in the perennial ryegrass bioassays. The effluent in these tubes was freeze dried (VirTis Company, Inc., Gardiner, NY) and subjected to the following purification steps

Twelve milligrams of 53GH-g15-e collected from the gel filtration column was subjected to a high performance liquid chromatography (HPLC) system (ISCO Inc., Lincoln, NE) with a Dynamax C18 reversed phase (C18 RP), 250- x 10-mm inner diameter, 5-µm particle size, 100 Å pore size, column (Rainin, Woburn, MA). A methanol (MeOH) in water linear gradient was used, with 0-25% MeOH in 50 min, 25-100% MeOH in 25 min, maintained at 100% MeOH I5 min, and 100-0% MeOH in the next 10 min at flow rate of 4 mL/min. The sample was monitored by UV absorbance at 280 nm with 0.02 absorbance units of full scale (AUFS). Fractions were collected at 10-min intervals for the first 50 min and at 25-min intervals for the next 50 min. Seven fractions were collected and subjected to the perennial ryegrass bioassay to determine their bioactivity. Four fractions were found to have greater than 90% root inhibition. The third fraction (GH-hplcfrn3) was chosen for further purification in this study.

A volume of 150 µL of aqueous solution containing 12 ma of the bioactive 53GH-g15-e was injected onto a semipreparative Delta Pak C18 RP, 300- x 7.8-mm inner diameter, 5 µm, 100 Å, column (Waters Chromatography Division, Millipore, MA) with a MeOH in water lin-

ear gradient of from 0 to 15% in 30 min at a flow rate of 4 mL/min. The sample was monitored by UV absorbance at 214 nm with 1.0 AUFS. Five fractions were collected based on the five major peaks with retention time (Rt) between 19 and 29 min on the chromatogram. The same peaks were pooled from 18 consecutive runs and were freeze dried for a modified perennial ryegrass bioassay. Half of a 7-cm diameter Whatman no. I filter paper and 0.5 mL of aqueous solution were used for the bioassay. Five perennial ryegrass seeds were placed on the wetted filter paper.

One peak (GH-hplc-pk4) of the five peaks was identified as bioactive and subjected to further purlfication by using a C18 RP narrow bore, 250- x 2.1-mm inner diameter column (Vydac, Hesperia, CA) with a linear AB gradient from 5 to 25% B in 40 min, where eluent A was 0.1% trifluoroacetic acid (TFA) in D.D. H$_2$O and B was 0.08% TFA in acetonitrile at a flow rate of 200 µL/min. A major peak was isolated and subjected to peptide sequencing on a Biosystems 477A Protein Sequencer (Applied Biosystems Inc., Foster City, CA) with a 120A PTH amino acid analyzer equipped with a PTH, 200- x 2.1-mm inner diameter, 5-µm column (Applied Biosystems Inc.). Determination of the amino acid composition was done with the Applied Biosystems model 420A derivatizer, model 130A separation system with 220- x 2.1-mm inner diameter, 5-µm PTC column, and a model 920A data analysis system. The molecular weight was determined on a Laser Desorption Mass Spectrometer (Finnigan MAT, San Jose, CA).

Validation of the Inhibitory Activity of the Pure Compound

To confirm the bioactivity of the isolated compound, peptide with the same sequence as the bioactive peak isolated from CGH was synthesized by the Protein Facility at Iowa State University and was tested for its inhibitory activity. An aqueous solution in a concentration series of 0, 0.5, 1.0, 1.5, 2.0, and 4.0 mg/mL was prepared and subjected to the perennial ryegrass bioassay

Statistical Analysis

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

Results and Discussion

The filtrate of gluten hydrolysate aqueous solution (GHf) inhibited root growth of germinating perennial ryegrass seeds at lower concentrations than that required to inhibit shoot growth (Fig. 1). Root length was inhibited by 50% at a concentration of 1.2 mg/mL in the Petri dish bioassay. It required approximately 2.8 mg/mL of filtrate to have 50% of shoot length inhibition.

From the calibration profile of the gel filtration column, the blue dextran was collected in tube 30, and the conductivity measurement dropped to zero at tube 60, indicating that tubes 30-60 were within the included volume (Fig. 2). Among the 80 fractions collected from the gluten hydrolysate filtrate eluted through Sephadex G-15 column, samples in fractions 50-56 (50GH-g15-e to 56GH-g15-e) completely inhibited both root and shoot growth of perennial ryegrass (Fig. 2). Materials in tubes
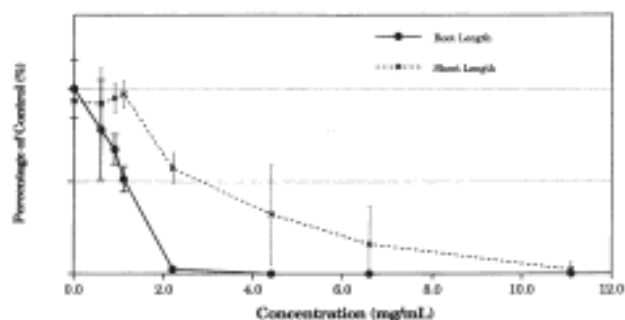
Fig. 1. The effect of gluten hydrolysate aqueous filter (GH-f) on seedling growth of germinating perennial ryegrass seeds tested in the growth chamber. Ten seeds were placed on one layer of 7-cm-diameter Whatman no. 1 filter paper containing 1 mL of test solution in each Petri dish. All dishes were incubated for 7 days in the growth chamber with 90 μmol/s/m^2 fluorescent lighting at 25°C for 8 h, 15°C dark for 16 h. The control had a root length of 45 $\pm$ 7 mm and a shoot length of 36 $\pm$ 4 mm (n = 7). The LSD$_{0.05}$ were 13.4 and 17.5%, respectively. Error bars indicated one S.D. for each treatment.

Fig. 3. Elution profile and fractionation of bioactive fraction collected from a Sephadex G-15 column on a C18 RP HPLC column. Column: Dynamax C18 RP 250- x 10-mm inner diameter. Conditions: linear AB gradient from 0 to 25% B in 50 min, 25 to 100% B in 25 min, 100% B in 10 min, and 100 to 0% B in 10 min, where eluent A is D.D. H$_2$O and B is 100% MeOH; flow rate of 4.0 mL/min. Detection: UV at 280 nm, 0.02 AUFS. Sample = 12 mg of 53GH-g15-e. Seven fractions were collected.
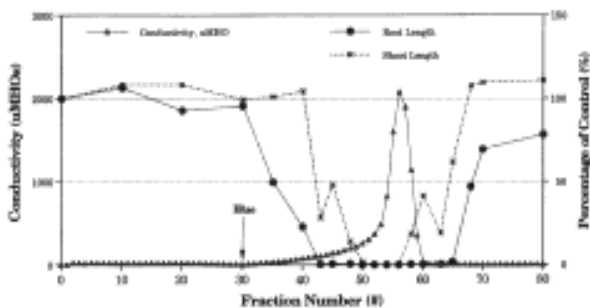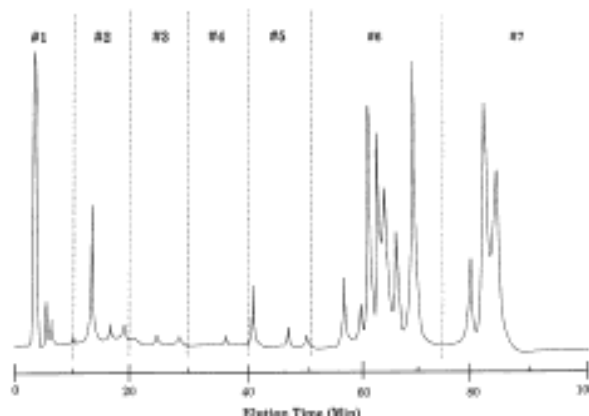

Fig. 2. Calibration of the Sephadex G-15 column and bioassay results for selective fractions of GH-g15-e. Blue dextran and NaCl dissolved in D.D. H$_2$O were loaded onto a 28- x 998-mm Sephadex G-15 column and eluted with D.D. H$_2$O at a flow rate of 1.6 mL/min. Eighty fractions with 270 drops of effluent per fraction were collected, and conductivity was measured. Thirty milliliters of 10% gluten hydrolysate filtrate (GH-f) 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 1. The control had a root length of 42 $\pm$ 10 mm and a shoot length of 34 $\pm$ 5 mm (n = 7). The LSD$_{0.05}$ were 13.4 and 12.4% for root length and shoot length, respectively.

43-49 and 57-65 completely inhibited roots, but shoots were reduced less than 50%. This study focused on the isolation of the compound coeluted with the five dipeptides found earlier from the gel filtration column (Liu and Christians 1994). Therefore, 53GH-g15-e, which completely inhibited root and shoot growth of germinating perennial ryegrass, was chosen for further purification.

The effluent in tube 53 (53GH-g15-e) subjected to a HPLC separation procedure with a 100-min run time was fractionated into seven parts (Fig. 3). Among the seven

fractions, fractions 1 (GH-hplc-frn1) and 3 (GH-hplcfrn3) had the most root-inhibiting activity in the perennial ryegrass bioassay (Fig. 4). Five dipeptides were isolated from GH-hplc-frn1 (Liu and Christians 1994). Fraction 3 (GH-hplc-frn3), which was eluted with 10-15% MeOH, inhibited 95% of root growth of germinating perennial ryegrass compared with the control (Fig. 4). More peaks could be detected at UV 214 nm (Fig. 5) with the same sample eluted with same gradient range than at UV 280 nm (Fig. 3). Five distinct peaks were eluted with 10-15% MeOH gradient and were collected from the HPLC effluent. This step was repeated 18 times, and each of the five fractions was pooled to have enough material for the bioassay. Peak 4 (GH-hplc-pk4), with a Rt of 25.5 min, was the most inhibitory to the root growth of germinating perennial ryegrass (Fig. 6). This bioactive peak (GH-hplc-pk4) was purified further using a narrow bore column and found to be a five-residue peptide, Leu-Ser-Pro-Ala-Gln.

The bioactivity of the pentapeptide was validated by testing a synthetic peptide with the same sequence as the identified peptide. The pentapeptide showed inhibitory activity to both root and shoot growth of germinating ryegrass (Table 1). At a concentration of 2.0 mg/mL, 90% of root growth and 82% of shoot growth were inhibited, and at 4 mg/mL, the seeds were essentially killed. This compound inhibited 50% root growth at concentration of 0.5 mg/mL in the perennial ryegrass bioassay. Therefore, its root-inhibiting activity on a weight basis was as active as Ala-Ala, the most active dipeptide isolated from corn gluten (Liu and Christians 1994). However, the molecular weight is 514.56 for the pen-
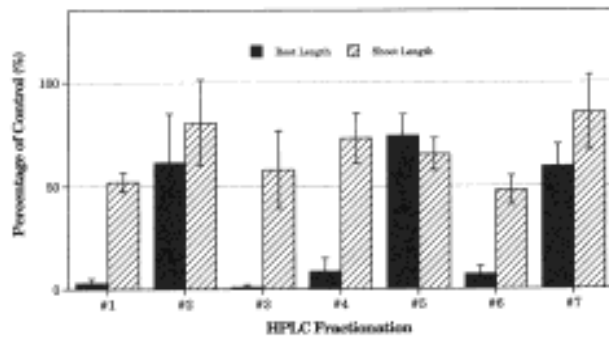
Fig. 4. Bioactivity of fractions of gluten hydrolysate collected from HPLC effluent tested by perennial ryegrass bioassay in the growth chamber. Fractionation conditions are given in Figure 3. The bioassay conditions were the same as in Figure 1. The control has a root length of 48.7 ± 9.9 mm and a shoot length of 45.9 ± 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 one S.D. for each treatment.

Fig. 6. Bioactivity of fractionations of 53GH-gl5-e gluten hydrolysate collected from HPLC effluent determined by using perennial ryegrass bioassay. Data were generated from the combination of 18 HPLC runs. Five seeds placed on half of one layer of 7-cm-diameter Whatman no. 1 filter paper containing 0.5 mL/Petri dish. Control had root length of 29.3 ± 2.9 mm, and shoot length of 22.7 ± 2.3 mm ($n = 3$). The $LSD_{0.05}$ were 34.9 and 26.6% for root length and shoot length, respectively. Error bars indicate one S.D. for each treatment. Fraction 4 was the peak of Rt = 25.3 min.
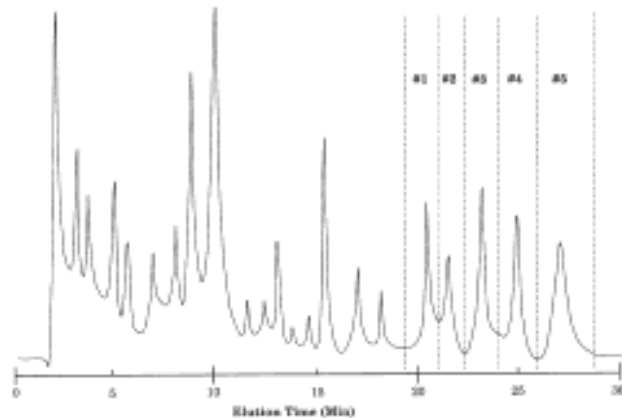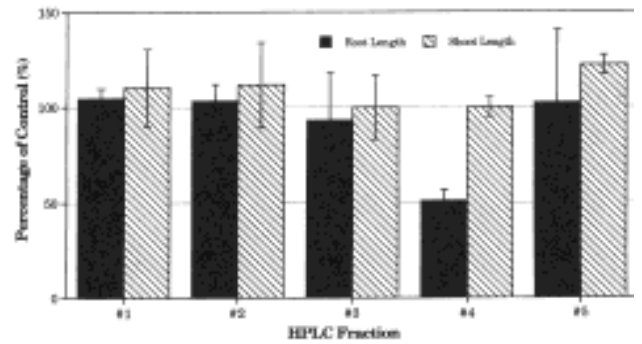

Fig. 5. Elution profile and fractionation of bioactive fraction collected from a Sephadex G-15 column, on a C18 RP HPLC column. Column: Waters C18 RP 300- x 7.8-mm inner diameter. Conditions: linear AB gradient from 0 to 15% B in 30 min, where eluent A is D.D. H_2O and B is 100% MeOH; with a flow rate of 4.0 mL/min. Detection: UV at 214 nm, 1.0 AUFS. Sample = 12 mg of 53GH-gl5-e. Five fractions were collected.

Table 1. Bioactivity of Leu-Ser-Pro-Ala-Gln in perennial ryegrass Petri dish bioassay

Concentration (mg/mL)	Percentage of control[a] (%)	
	Root length	Shoot length
0.5	50.1 ± 16.9	14.5 ± 3.2
1.0	44.2 ± 17.6	15.5 ± 2.7
1.5	27.7 ± 7.1	11.6 ± 2.7
2.0	10.2 ± 9.1	13.5 ± 4.4
4.0	0	0.5 ± 1.3

Note. Each dish contained a Whatman no. 1 filter paper measuring 38.5 cm^2 in area.
[a] Control had a root length of 39 ± 7 mm and a shoot length of 30 ± 3 mm ($n = 7$). Values are means of measurement of the length of seven seedlings $\pm$ S.D. The $LSD_{(0.05)}$ were 14.1 and 4.9%, respectively.

tapeptide versus 160.2 for Ala-Ala. Therefore, the root inhibitory activity of the pentapeptide is greater than that of the dipeptides on a molar basis.

The authors have suggested that there may be more than one bioactive compound in the CGH aqueous solution (Liu and Christians 1994). In this study, we found another peptide that is more hydrophobic and more inhibitory to root formation of perennial ryegrass than the five dipeptides found earlier. The bioassay results of the fractions collected from gel filtration suggested that there are probably at least two other groups of compounds in CGH hydrolysate which have root inhibiting activity. All six bioactive peptides isolated from the aqueous solution

of CGH were proved to inhibit root formation at a concentration lower than that of either CGM or CGH, and they were demonstrated as naturally occurring, growth regulating compounds. They have potential for use in combination with nontoxic, compatible carriers or for the fortification of the CGM or CGH to increase their effectiveness as preemergence herbicides. Field studies using CGM and CGH to develop as natural preemergence herbicides in turf and strawberry plots are under way.

References

Abacus Concepts (1991) StatView. Abacus Concepts, Berkeley, CA

Christians NE (1991) Preemergence weed control using corn gluten meal. US patent 5,030,268

Christians NE (1993) The use of corn gluten meal as a natural preemergence weed control in turf. In: Carrow RN, Christians NE, Shearman RC (eds) International Turfgrass Society Research Journal. Intertec Publishing, Overland Park, KS, pp 284-290

Christians NE, Garbutt JT, Liu DL (1994) Preemergence weed control using plant protein hydrolysate. US patent 5,290,749

Lax AR, Shepherd HS, Edwards JV (1988) Tentoxin, a chlorosis inducing toxin for *Alternaria* as a potential herbicide. Weed Technol 2:540-544

Liu DL, Christians NE (1994) Isolation and identification of root inhibiting compounds from corn gluten hydrolysate. J Plant Growth Regul 13:227-230

Liu DL, Christians NE, Garbutt JT (1994) Herbicidal activity of a hydrolysed corn gluten meal on three grass species under controlled environments. J Plant Growth Regul 13:221-226

Lydon J, Duke SO (1987) Progress toward natural herbicides from plants. Herbs Spices Med Plants Dig 5:1-

Nonnecke GR, Christians NE (1993) Evaluation of corn gluten meal as a natural weed control product in strawberry. Acta Hortic 348: 315-320

Snedecor GW, Cochran WG (1989) Statistical method, 8th ed. Iowa State University Press, Ames, IA