

Isolation and Identification of Root-Inhibiting Compounds from Corn Gluten Hydrolysate

Dianna Lan-Ying Liu and Nick E. Christians *

Department of Horticulture, Iowa State University, Ames, Iowa 50011, USA
Received December 3, 1993; accepted August 19, 1994

Abstract. 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. Corn gluten hydrolysate, found to have a growth-regulating effect on the root system of germinating grass seeds, has been suggested as a natural herbicide. A protocol was developed to extract, isolate, and identify the root-inhibiting compounds from corn gluten hydrolysate aqueous solution and a perennial ryegrass (*Lolium perenne* L.). A Petri dish bioassay was used to test the root-inhibiting activity. Five bioactive dipeptides were isolated by using Sephadex G-15 gel filtration, solid-phase extraction, and C18 reversed-phase high-performance liquid chromatography procedures. The five dipeptides were glutaminyl-glutamine, alaninyl-asparagine, alaninylglutamine, glyciny-alanine, and alaninyl-alanine. Their root-inhibiting activity on perennial ryegrass was demonstrated in Petri dish bioassays.

Although chemicals with increased efficacy and safety have been developed in recent years, the agrochemical industry faces growing criticism over the toxicity and residue problems associated with the use of pesticides (Huppatz 1990). Because many pesticides are toxic or biologically active by design, the use of synthetic pesticides for weed control has become a serious concern (Balogh and Anderson 1992). The judicious application of herbicides has become an integral part of agriculture (Pimentel 1986). Some natural plant compounds that inhibit growth and development of other plants may function as herbicides or serve as the starting point for

the chemical synthesis of biodegradable herbicides. Interest has centered on the use of plant-derived compounds as natural herbicides, and they may represent an environmentally sound approach to weed control (Lax et al. 1988, Lydon and Duke 1987, Rice 1984).

It has been reported that corn gluten meal, which is a byproduct of corn from the wet-milling process, is useful as a natural pre-emergence herbicide and fertilizer for various plant production systems (Christians 1993). It could inhibit root growth of germinated seed, but no damage was observed to plants with a mature root system. Corn gluten hydrolysate, a water-soluble material derived from the action of bacterial proteinase, was demonstrated to be more herbicidally active to three grass species (Liu et al. 1994) than corn gluten meal.

If the bioactive compound in corn gluten hydrolysate can be isolated and identified, it may be used as natural herbicide or serve as a starting point for chemical synthesis of biodegradable herbicides. The objective of this study was to develop a protocol to extract, isolate, and identify the root-inhibiting compounds from corn gluten hydrolysate.

Materials and Methods

Perennial Ryegrass Bioassay

Ten perennial ryegrass (*Lolium perenne* L.) seeds were placed on one layer of 7-cm-diameter Whatman No. 1 filter paper, measuring 38.5 cm², in a 9-cm-diameter Petri dish. One milliliter of test solution was applied to each Petri dish. The dish was covered with a lid, sealed with Parafilm[®], and placed in a controlled environmental chamber. The radiation intensity in the growth chamber was at 70 $\mu\text{mol/s/m}^2$ 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 was measured to the nearest millimeter, and the measurements of seedlings from the same dish were averaged. Seven of the 10 seedlings in each Petri dish found to have root lengths within ± 1 SD were selected and averaged. The seven measurements of each treatment were di-

vided by the root length from the control plate that had only the deionized distilled water (D.D. H₂O) in filter paper, multiplied by 100, and expressed as a percentage of control length (%). The amount of material required to inhibit 50% of the control length was defined as 1 unit of activity.

Extraction and Purification

Gluten hydrolysate powder was dissolved in D.D. H₂O to make a 10% solution. This aqueous solution of gluten hydrolysate was designated GH1. A dilution series of GH1 was prepared for the perennial ryegrass bioassay. For bioassay use, the GH1 was filtered through a 0.2- μ m membrane filter (German Sciences Inc., Ann Arbor, MI, USA) to remove particles. The filtrate was designated GH7. A dilution series of GH7 was made for the perennial ryegrass bioassay.

Thirty milliliters of GH7 were loaded onto a 28 x 998-mm (bed volume = 630 mL) Sephadex G-15 (Sigma Chemical Company, St. Louis, MO, USA) gel-filtration column. The column was eluted with D.D. H₂O at a flow rate of 1.6 mL/min. The effluent (GH17) was collected in 80 tubes with 270 drops in each tube by using a Gilson Model 201 fraction collector (Gilson Medical Electronics, Inc., Middleton, WI, USA). Selected tubes were bioassayed for the root-inhibiting bioactivity by using the perennial ryegrass bioassay as described. Bioassay results showed that tubes 50-56 (50GH17 to 56GH17) contained the root- and shoot-inhibiting compound(s). These tubes (GH17s) were freeze-dried and subjected to further purification steps.

The bioactive 53GH17 was passed through C18 Sep-Pak cartridges (Waters Chromatography Division, Millipore Corporation, MA, USA), and the fraction, designated GH27-1, that was not trapped by the cartridge was collected. A dilution series of GH27-1 was made for the perennial ryegrass bioassay.

Six milligrams of GH27-1 were injected onto the C18 reversed-phase high-performance liquid chromatography (C18-RP HPLC; 250 x 10 mm I.D.) column (Rainin) with methanol (MeOH) in water (linear gradient of 0-5% in 10 min) at a flow rate of 4 mL/min. The sample was monitored by UV absorbance at 214 nm with 1.0 absorbance unit full scale (AUFS). Fractions were collected based on the major peaks of the chromatogram. The same peaks were pooled from six consecutive runs for the perennial ryegrass bioassay.

One (GH47-4) of the 10 peaks was identified as bioactive and subjected to amino acid analysis and peptide sequencing, conducted by the Protein Facility at Iowa State University. Determination of the amino acid composition was done with the Applied Biosystems Model 420A derivatizer, Model 130A separation system (220 x 2.1 mm i.d., 5 μ PTC column), and a Model 920A data-analysis system (Applied Biosystem, Inc., Foster City, CA, USA).

Validation of the Inhibitory Activity of the Pure Compounds

To confirm the root-inhibiting activity of the five compounds identified by the ISU Protein Facility, five synthetic pure dipeptides, glutamyl-glutamine (Gln-Gln; BACHEM Bioscience, Inc., Philadelphia, PA, USA), alaninyl-asparagine (Ala-Asn), alaninyl-glutamine (Ala-Gln), glycyl-alanine (Gly-Ala), and alaninyl-alanine (Ala-Ala), were obtained commercially (Sigma). Gln-Gln, Ala-Asn, Ala-Gln, Gly-Ala, and Ala-Ala were prepared in aqueous solutions with a concentration series of 0, 0.3, 0.5,

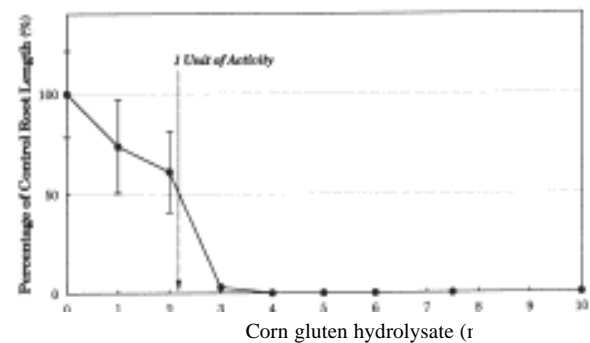


Fig. 1. The effect of gluten hydrolysate aqueous solution (GH1) on radicle growth of germinating perennial ryegrass seeds tested in the growth chamber. Ten seeds were placed on one layer of 7-cm-thick Whatman #1 filter paper containing 1 mL of test solution in each Petri dish. All dishes were incubated for 10 days in the growth chamber with 70 μ mol/s/m² fluorescent lighting at 25°C for 16 h and dark for 8 h at 15°C. The control had a root length of 34 \pm 7 mm (n = 7). The LSD (0.05) was 13.4%. Error bars indicate 1 SD for each treatment.

0.8, 1.0, 1.2, 1.5, and 2.0 mg/mL and subjected to the perennial ryegrass bioassay.

The bioactive dipeptides and purification subsamples were tested by using the perennial ryegrass bioassay in three replicates to compare the unit of activity of each sample. Each of the samples was dried by using a Model 10-100 Virtis freeze dryer (Virtis Company Inc., Gardiner, NY, USA), and dry weights were measured.

Statistical Analysis

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

Results and Discussion

A dose-response curve of gluten hydrolysate aqueous solution (GH1) demonstrated that 2.2 mg of gluten hydrolysate inhibited 50% of the root length as compared with the control, thus providing 1 unit of activity (Fig. 1). Among the 80 fractions (GH17s) collected from gluten hydrolysate filtrate (GH7) eluted through the Sephadex G-15 column, fractions 50GH17-56GH17 completely inhibited both root and shoot growth of perennial ryegrass (Fig. 2). Fractions 43GH17-49GH17 and 57GH17-65GH17 completely inhibited roots, but shoot length had greater than 50% reduction. The bioassays suggest that there may be more than one bioactive compound in the corn gluten hydrolysate aqueous solution, or that the root systems of perennial ryegrass

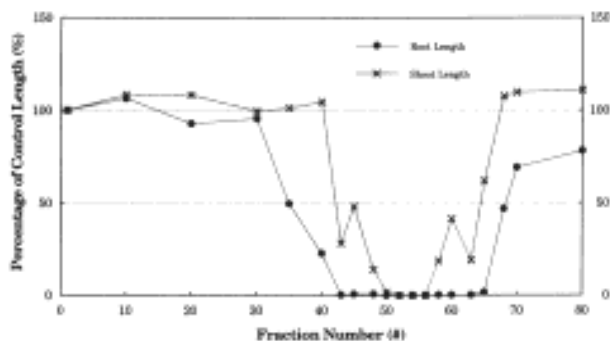


Fig. 2. Bioassay results for selective fractions of GH17s collected from a Sephadex G-15 column. The control had a root length of 42 ± 10 mm and a shoot length of 34 ± 5 mm ($n = 7$). The LSD (0.05) were 13.4% and 12.4% for root length and shoot length, respectively.

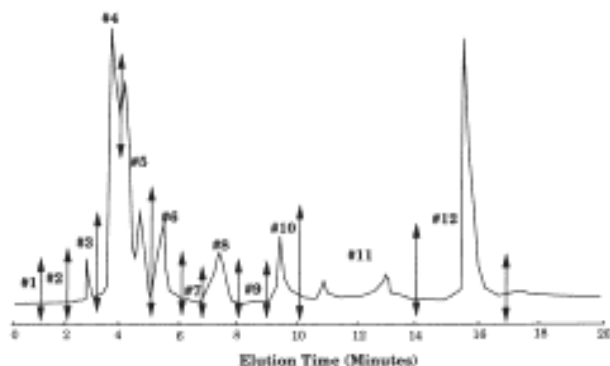


Fig. 3. Elution profile and fractionation of GH27-1, flow-through from a C18 Sep-Pak cartridge, on reversed-phase C18 HPLC column. Column: Dynamax C18-RP 250 x 10.0 mm I.D. (Rainin). Conditions: linear AB gradient from 0 to 5% B in 10 min, 5 to 100% B in 2 min, 100% B for 7 min, and 100 to 0% B in 1 min, where eluent A is D.D. H₂O and B is 100% MeOH; flow-rate, 4.0 mL/min. Detection: UV at 214 nm, 1.0 AUFS. Sample: 6 mg of 53GH27-1. Twelve fractions were collected.

are inhibited at lower concentrations of a single bioactive compound than are the shoots.

Tube 53, collected from the gel-filtration column (53GH17s), was subjected to a C18 Sep-Pak cartridge before the HPLC separation procedure and the bioactive flow-through; 53GH27-1 (data not shown) was then loaded on to a semipreparative C18-RP HPLC column (Rainin), and monitored at UV 214 nm (Fig. 3). Twelve fractions were collected from the HPLC effluent. This step was repeated six times, and each of the 12 fractions was pooled to have enough material for the bioassay. Fraction 4 (GH47-4), which corresponded to the second major peak in Fig. 3, was the most inhibitory, followed by fraction 5 (Fig. 4).

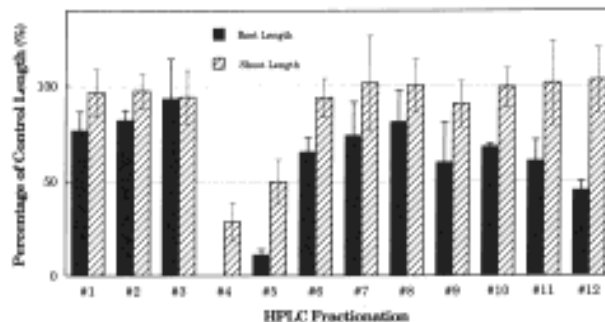


Fig. 4. Bioactivity of fractions of GH47-4 collected from the HPLC effluent (GH47) by using the perennial ryegrass bioassay in the growth chamber. Data were generated from the combination of six HPLC runs on a Dynamax C18-RP 250 x 10.0 mm I.D. column; fractionation method is described in Fig. 3. The control had a 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 SD for each treatment.

Table 1. Comparison of root-inhibiting activity among subsamples of corn gluten hydrolysates and dipeptides expressed as the amount of material required to inhibit 50% of the control root length determined from the dose-response curve of each sample..

Test sample	1 Unit of activity (mg) ^a
GH1	1.6 ± 0.2
GH7	1.3 ± 0.1
GH17	1.2 ± 0.1
GH27-1	1.1 ± 0.1
GH47-ACPK	1.1 ± 0.1
Gln-Gln	1.1 ± 0.1
Ala-Asn	0.9 ± 0.1
Ala-Gln	0.8 ± 0.1
Gly-Ala	0.5 ± 0.1
Ala-Ala	0.5 ± 0.1

^a Values are means of the replications \pm SE. Each sample had three replicates, except GH1, which had four replicates. The LSD (0.05) was 0.4 mg.

The GH47-4 was subjected to amino acid analysis and peptide sequencing. The GH47-4 was identified as a combination of the following dipeptides: Gln-Gln, Ala-Asn, Ala-Gln, Gly-Ala, and Ala-Ala.

The five identified dipeptides and samples of partially purified corn gluten hydrolysate were compared for their ability to inhibit root growth of perennial ryegrass seedlings (Table 1). The crude extract, GH1, required 1.6 ± 0.2 mg to achieve 1 unit of activity. This was significantly greater than that of all the other samples except GH7. The unit activity of the active peak isolated from C18-RP HPLC (GH47-ACPK) was 1.1 ± 0.1 mg, which was significantly greater than that of Gly-Ala and Ala-Ala, but not that of the other dipeptides. Among the



Fig. 5. Protocol for isolating root-inhibiting compounds from corn gluten hydrolysate.

five dipeptides, Ala-Ala and Gly-Ala were the most bioactive, and Gln-Gln was the least bioactive, for root-inhibition. The procedures developed in this study (Fig. 5) were able to isolate root-inhibiting compounds from corn gluten hydrolysate aqueous solution.

The herbicidal activity of corn gluten hydrolysate has been demonstrated elsewhere (Liu and Christians 1993). This study showed that more active compounds could be isolated from corn gluten hydrolysate. The five dipeptides isolated from the aqueous filtrate of the gluten hydrolysate root formation of germinating perennial ryegrass, demonstrating their potential for use as naturally occurring, growth-regulating compounds and as natural pre-emergence herbicides. The naturally occurring, bioactive dipeptides could be used in combination with nontoxic, compatible carriers, including water and clay for use in agriculture. They might also be

used to fortify the corn gluten or gluten hydrolysate to increase their effectiveness as selective pre emergence herbicides. Field studies are planned

Acknowledgments. We thank the Grain Processing Corporation, Muscatine, Iowa, for providing financial assistance and samples of corn gluten hydrolysate. We are grateful to the staff of the Protein Facility at Iowa State University for help in peptide sequencing and amino acid analysis.

References

- Balogh JC, Anderson JL (1992) Environmental impacts of turfgrass pesticides. In: Balogh JC, Walker WJ (eds) Golf course management and construction—Environmental issues. Lewis Publishers, Chelsea, pp 221-354
- 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, pp 284-290
- Hupatz JL (1990) Essential amino acid biosynthesis provides multiple targets for selective herbicides. In: Casida JE (ed) Pesticides and alternatives: Innovative chemical and biological approaches to pest control. Elsevier, New York, pp 563-572
- Lax AR, Shepherd HS, Edwards JV (1988) Tentoxin, a chlorosis-inducing toxin from *Alternaria* as a potential herbicide. *Weed Technol* 2:540-544
- Liu DL-Y, Christians NE, Garbutt JT (1994) Herbicidal activity of a hydrolyzed corn gluten meal on three grass species under controlled environments. *J Plant Growth Regul* 13: 00-00
- Lydon J, Duke SO (1987) Progress toward natural herbicides from plants. *Herbs Spices Med Plants Dig* 5:1-4
- Pimentel D (1986) Status of integrated pest management. In: Pimentel D (ed) Some aspects of integrated pest management. Cornell University Press, Ithaca, pp 29-68
- Rice EL (1984) Allelopathy, 2nd ed. Academic Press, New York
- Snedecor GW, Cochran WG (1989) Statistical methods, 8th ed. Iowa State University Press, Ames