Isolation of a Biologically Active Substance from Rhizomes of Quackgrass [*Elymus repens* (L.) Gould]

Siegfried A. Korhammer* and Ernst Haslinger

Abteilung Organische Chemie I, University of Ulm, D-89069 Ulm, Germany

Quackgrass [*Elymus repens* (L.) Gould] seems to possess allelopathic properties. The substances responsible for this are still unknown. A substance having strong inhibitory effects on the germination of seeds of alfalfa at concentrations lower than 10^{-6} mg/mL of water has been isolated from rhizomes of quackgrass. It was isolated using reversed-phase chromatography, gel permeation chromatography, and ion-exchange chromatography and was identified by mass spectrometry and NMR as indole-3-acetic acid.

Keywords: *Quackgrass; Elymus repens (L.) Gould; Agropyron repens (L.) P. Beauv.; allelopathy; indole-3-acetic acid*

I. INTRODUCTION

Quackgrass [*Elymus repens* (L.) Gould, *Triticum repens* L., *Agropyron repens* (L.) P. Beauv., *Elytrigia repens* (L.) Nevski] is a widespread perennial grass that reproduces mainly by rhizomes (Werner and Rioux, 1977). Since 1912 numerous studies have demonstrated its deleterious effects on other plants. Most of the authors report on the allelopathic or phytotoxic activity of quackgrass, but the results and interpretations are contradictory (Hamilton and Buchholtz, 1955; Knapp, 1980; Kommedahl et al., 1970; Kraus, 1912; Penn and Lynch, 1982; Plhák, 1967; Toai and Linscott, 1979; Weinberger, 1963; Welbank, 1963).

Even though there are many assumptions about the nature of the allelochemicals (Gabor and Veatch, 1981; LeFevre and Clagett, 1960; Osvald, 1948; Plhák and Helan, 1965; Weston et al., 1987) and Tauscher (1982) identified some biologically active compounds, there are only three structurally defined allelochemicals isolated from quackgrass. Weston et al. (1987) isolated 5,7,4′-trihydroxy-3′,5′-dimethoxyflavone (tricin) from quackgrass shoots, and Hagin (1989) isolated 5-hydroxyindole-3-acetic acid (5-HIAA) and 5-hydroxytryptophan (5-HTP) from quackgrass roots and rhizomes. We subjected these substances to a germination test using seeds of alfalfa ($1.0-2 \times 10^{-6}$ mg/mL of water). Tricin showed no effects, whereas 5-HIAA and 5-HTP inhibited seed germination, but only at very high concentrations (1.0–0.25 mg/mL of water). Weston et al. (1987) and Hagin (1989) found effects on seedling growth. Tricin was synthesized (Anderson, 1933; Gaydou and Bianchini, 1978) and purified by HPLC; 5-HIAA and 5-HTP were obtained from Janssen and used without further purification.

The aim of this work was to isolate substances from quackgrass which may have allelopathic properties.

II. MATERIALS AND METHODS

1. Quackgrass Material. We used for our work the subterraneous parts of quackgrass (rhizomes). We have collected them from sods near Bayreuth (April–October 1990) and Ulm (May 1991), Germany. Voucher specimens are deposited at the University of Bayreuth, Ökologisch-Botanischer Garten. After the rhizomes were washed with water and air-dried, they were shock frozen by immersion in liquid nitrogen and later ground using a mill. The material (about 12 kg) was stored at −20 °C.

2. Sample Extraction and Purification. Organic solvents (analytical grade) were removed using a rotary vacuum evaporator (water bath set at 40 °C; before getting dry, water was added to such fractions that did not contain any water), water (double-distilled) was removed using freeze-drying.

After extraction of the rhizomes with methanol/water (3:1; 9 L/300 g, 12 h, stirring at room temperature) and filtering using a Büchner funnel and centrifugation, the methanol was removed. Then acetone was added to the aqueous solution (4:1). The mixture was stirred for 12 h at room temperature. After decantation, the acetone was removed and the solution was lyophilized. In the bioassay the precipitate did not show any activity, whereas the freeze-dried aqueous extract was active. Five milligrams was used for the test. We received 750 g of active extract. It was concentrated using several chromatographic methods. The fractions obtained by the chromatographic techniques were tested by the bioassay. Those showing inhibitory activity were combined and subjected to the following separation step.

2.1. Flash Chromatography (FC). 2.1.1. FC on RP-18. Up to 60 g of lyophilized extract was dissolved in water and put on the column (5.0 × 80 cm) which was filled with 12 cm of LiChroprep RP-18 (40–63 μm, Merck, conditioned with water). After washing with water, methanol was added until the eluate became green. Then it was washed with diethyl ether. The water eluate was discarded. The bioassay showed that the methanolic fraction was active. We used 2.0 mg of the residue for the test. We obtained 130 g of methanolic extract.

2.1.2. FC on RP-8. The dried active material was subjected to FC on RP-8 [column 5.0 × 80 cm, filled with 12 cm of LiChroprep RP-8 (40–63 mm, Merck), conditioned with water]. For each separation up to 10 g was dissolved in water. We used as eluting solvents 400 mL of methanol/water (1:1) and then 600 mL of methanol. The methanol/water eluate was active. Half a milligram was bioassayed. We obtained 45 g of active fraction.

2.2. Medium-Pressure Liquid Chromatography (MPLC). MPLC was performed using a Sepapress HPP-40/200 pump (Kronwald) and a refraction detector (Sepachrom RI-20, Kronwald).

2.2.1. MPLC on RP-8. Up to 1.2 g of the active fraction was dissolved in 2 mL of methanol and injected on the column (Sepakron HPP, series VPC-FPGC, Kronwald, 3.0 × 30 cm), filled with LiChroprep RP-8 [40–63 μm, Merck, conditioned with methanol/water (1:1)]. The flow rate was 30 mL/min.

* Address correspondence to this author at the Internationales Hochschulinstitut Zittau, Markt 23, D-02763 Zittau, Germany (fax 49/3583/771534).

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Three fractions were obtained: t (min) = 0–4.5, t = 4.5–5.5 (after drying, chromatographed again), t = 5.5 (changing to methanol as solvent)–20. The third fraction was active; 0.2 mg was used for testing the activity. We obtained 11.3 g of active material.

2.2.2. MPLC on RP-2. Up to 600 mg of the active fraction was dissolved in 2 mL of methanol and injected on the column (Sepakron HPP, series VPC, Kronwald, 1.5 × 20 cm) filled with LiChroprep RP-2 [25–40 μm, Merck, conditioned with methanol/water (1:2)]. The flow rate was 15.2 mL/min. Three fractions were obtained: t (min) = 0–3, t = 3–5 (after drying, chromatographed again), t = 5 (changing to methanol as solvent)–16. The first fraction was active. For the bioassay we used 0.1 mg. We obtained 6.64 g of active material.

2.3. Gel Permeation Chromatography (GPC). 2.3.1. GPC on Sephadex LH-20. Up to 250 mg of the active fraction was dissolved in up to 2 mL of methanol and subjected to the separation (column 2 × 85 cm, filled with Sephadex LH-20–100, Fluka, solvent methanol, flow rate ca. 12 mL/100 min). The fractions 150–200 mL were active. We used 0.05 mg for the germination test. We received 1.21 g of active material.

2.3.2. GPC on Sephadex G-10. Five milliliters of water was added to the active residue. The dissolved part was lyophilized. We obtained 230 mg, which was redissolved in water and then put on the column (2 × 85 cm, filled with Sephadex G-10, Sigma, solvent water, storage flask with 1 L of water, flow rate 12 mL/100 min). It decreased during the chromatography. To the water in the flask was added after 12, 24, and 36 h and after 60, 84, and 108 h 150 and 250 mL of methanol, respectively. After 132 h the solvent was changed to methanol. The fractions 68–71 were active; 0.1 mL of eluate from each fraction was used for the bioassay. We obtained 12 mg of active material.

2.4. High-Performance Liquid Chromatography (HPLC). HPLC was performed using a Beckman instrument (programmable solvent module 126 with two preparative pumps, 210A sample injection valve, diode array detector module 168). Precolumns were used. The separations were carried out at room temperature.

2.4.1. HPLC on RP-18. The active fraction was dissolved in water and injected (4 × 50 μL; column 0.46 × 25 cm, Ultrasphere ODS 5 μm, eluant A = water, adjusted with TFA to pH 2.8, eluant B = acetonitrile, flow rate 0.7 mL/min, λ = 284 and 354 nm). The following linear gradient was used: t (min) = 0–1, 15% B; t = 1–27; 15–67% B; t = 27–28, 67–100% B, t = 34–35, 100–15% B. The chromatogram (at λ = 284 nm) showed four main peaks at t_R (min) = 16, 17, 25, and 32. The fraction at t_R = 25 min showed a high level of biological activity. One one-hundredth of the total amount of each collected fraction was used for the bioassay. We obtained 4 mg of active material.

2.4.2. HPLC on TSK DEAE-2SW. The active fraction was purified using a Spherogel TSK DEAE-2SW column (0.46 × 25 cm, eluant A = water, eluant B = 0.1 M aqueous pyridine/acetic acid buffer, pH 5.1, flow rate 0.6 mL/min, λ = 268 and 354 nm, injection volume 50 μL). The following linear gradient was used: t (min) = 0–5, 90% B; t = 5–25, 90–100% B; t = 40–41, 100–90% B. The main fraction (t_R = ca. 30 min) was freeze-dried, redissolved in water, and then injected on a RP-18 column (Hypersil ODS 5 μm, 1.0 × 25 cm). After washing with water, the substance was eluted with acetonitrile. We obtained 3 mg of active substance.

3. Bioassay. For the identification of the biologically active fractions we used a bioassay. It was performed with seeds of alfalfa (*Medicago sativa* L.) because of the strong inhibitory effects of extracts of quackgrass on alfalfa (Kommedahl et al., 1959; Ohman and Kommedahl, 1960).

The amounts of the collected fractions were added as 1-mL methanolic aliquots to glass Petri dishes (5 cm i.d.), which were lined with two layers of filter paper. After evaporation of methanol at room temperature (the complete evaporation was checked with two control dishes containing 1 mL of methanol), 10 seeds were placed in each Petri dish. The filter paper was moistened with 1 mL of water, including two untreated controls. The dishes were deposited in a dark place at room temperature. After 24–72 h, the bioassays were analyzed. In active fractions the seeds showed diminished radicle growth or swelling only. The nonactive samples appeared before and after the group of samples showing inhibitory effects. This was a hint that only one compound would be responsible for the observed effects. The results were unambiguous, so no further investigations were made.

III. RESULTS AND DISCUSSION

The structure was determined by MS and ^{1}H NMR as 3-indoleacetic acid (IAA). The mass spectra (Varian MAT 711, EI, 70 eV, direct inlet 80–85 °C) showed two characteristic signals (m/z 175 and 130). The odd number indicated an uneven number of nitrogen atoms; the difference Δm = 45 was a hint for a COOH fragment. The integration of the 500-MHz ^{1}H spectra (AMX-500, Bruker, 5-mm tube, 0.5 mL of D_2O, 302 K) showed seven protons [δ = 3.63 (2H), 6.94, 7.02, 7.10, 7.27, 7.51]. Four of the aromatic protons formed an ABCD spin system. The signal at δ 3.63 did not show any coupling.

IAA, purchased from Fluka, showed the same NMR, MS, GC, and HPLC data, also in direct comparison with GC and HPLC analysis. TMS derivatives were used in GC. Furthermore, it showed the same effects in the bioassay.

IAA is especially known as a plant growth hormone, but it can also act as a plant growth inhibitor (Aberg, 1957). We tested the effect of different concentrations of IAA on the germination of seeds of alfalfa. In concentrations higher than 2 × 10^{-4} mg/mL of water, germination was totally inhibited. At a concentration of 2 × 10^{-6} mg/mL of water, germination was retarded. At concentrations lower than 2 × 10^{-8} mg/mL of water, germination was not disturbed.

In future studies the quantity of IAA released from quackgrass rhizomes, and its capacity to act as an allelochemical, should be investigated.

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