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COVALENTLY ATTACHED FERULIC ACID IN A PROTEOGLYCAN FROM RICE BRAN*

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Key Word Index—*Oryza sativa*, Gramineae, rice, hydroxyproline-containing proteoglycan, phenol derivatives, ferulic acid

Abstract—A water-soluble proteoglycan, precipitated with ammonium sulfate from the hot-water extract of rice bran, contained ferulic acid, which was liberated by alkaline treatment Evidence for the linkage between the carboxyl group of ferulic acid and the proteoglycan was obtained by the characterization of ferulic acid hydroxamate after treatment of the proteoglycan with hydroxylamine

INTRODUCTION

Previous studies have shown that the water-soluble, non-amylaceous polysaccharide from rice bran is a series of conjugated carbohydrates (proteoglycans) [1], and the structure of the carbohydrate–protein linkages was established as an *O*-α-L-arabinofuranosylhydroxyproline [2]

The protein content [1] of the proteoglycan fractions was measured by the method of Lowry *et al* [3] and elementary analysis There was no discrepancy between the protein contents of fractions B1–B6 measured by the two analytical methods, but there was with fraction A1 The method of Lowry *et al* [3] gave more protein than did elementary analysis The UV spectra of fractions B1–B6 had an absorption peak at 280 nm, while that of fraction A1 had absorption at 320 nm in addition to the absorption due to protein These preliminary results suggest that the proteoglycan Al contains a phenolic substance Previously, ferulic acid was found in rice (long-grain from Crowley and medium-grain from California), bran hemicellulose [4] and wheat flour hemicellulose [5], yet little is known of the binding between polymer and ferulic acid The results presented here demonstrate that ferulic acid is an integral component of proteoglycan A1

RESULTS

The ether extract (proteoglycan A treated by alkali) contained a phenolic substance, but the ethanol and ether extracts (sample untreated by alkali) did not (see Experimental) The phenolic was identified unambiguously as ferulic acid by standard procedures (see Experimental) The yield of ferulic acid was 0 3 mg per g proteoglycan A

Proteoglycan A contains a carbohydrate-free protein [1] DEAE-Sephadex-purified proteoglycan A (proteoglycan A1) [1] contained ferulic acid, but the contaminating protein did not

In order to determine the linkage of ferulic acid to proteoglycan A1, it was treated with hydroxylamine The hydroxamate was obtained by extraction with ethyl acetate and gel filtration on Sephadex LH-20 Gel filtration gave two peaks, the first due to hydroxylamine and the second due to ferulic acid hydroxamate Identification of the latter was based on comparison with authentic ferulic acid hydroxamate by PPC [6] and by UV and mass spectra (see Experimental) These results indicate strongly that the linkage between proteoglycan and ferulic acid is an ester linkage involving the carboxyl group of ferulic acid

DISCUSSION

Van Sumere *et al* [7] reported previously that *N*-feruloylglycyl-L-phenylalanine is obtained from barley globulins by partial hydrolysis with 4 M hydrochloric acid After our alkali-treated proteoglycan solution was extracted with ether, hydroxylamine treatment of the water phase did not release ferulic acid hydroxamate Therefore, it seems that an alkali-stable bound ferulic acid, as in barley globulin, does not occur in this proteoglycan In order to investigate the attachment of ferulic acid to the carbohydrate or protein moiety, enzymatic degradation of proteoglycan A1 was carried out, and the products were gel-filtered on Sephadex G-25 Hemicellulase [2] caused a decrease in the ability of formation of the hydroxamate of the high MW-fraction (effluents emerging at void volume), but protease [2] did not This preliminary experiment suggests that the ferulic acid is most probably linked to carbohydrate rather than to protein within proteoglycan A of rice

Recently, Uchiyama *et al* [8] found that the amount of ferulic acid increases in rice callus cell wall after inoculation of *Aspergillus oryzae* It is well known that phenols accumulate in infected cells [9] and that some of these compounds are toxic to certain plant pathogens The deposition of toxic ferulic acid and possibly other phenolic substances in the cell wall might be not only an important defence reaction [8], but also an alternative way of detoxication of these substances in certain plants, the formation of simple glucoside being the most common way [10] The chemical characteristics of carbohydrate

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and protein in this proteoglycan are very similar to those in plant cell walls [11] in the following respects (1) *O*-α-L-arabinofuranosylhydroxyproline forms an attachment between carbohydrate and protein, (2) the component sugars of the carbohydrate moiety are similar, and (3) the component amino acids, such as hydroxyproline of the protein moiety, are similar Ferulic and *p*-coumaric acids have been reported to be esterified to cell-wall components [12–14] Hartley *et al* [13, 14] discussed that the function of the phenolic substances of the esters could be to act as intermediates in the process of lignification Thus this proteoglycan may play an important role in the plant physiology of rice cell wall

EXPERIMENTAL

Plant material Bran of rice (*Oryza sativa*) was used

Isolation of proteoglycan Extraction of rice bran with hot H_2O followed by the removal of protein and starch yielded a proteoglycan Fractionation by salting out with saturated ammonium sulfate gave two fractions, namely proteoglycans A (ppt) and B (supernatant) Further fractionation by DEAE-Sephadex chromatography yielded several fractions (proteoglycans A1 and B1–B6) which were shown to be homogeneous by disc electrophoresis [1]

Detection of a phenolic substance from proteoglycan A Proteoglycan A, dissolved in deionized H_2O (1% w/v), was precipitated with EtOH The EtOH phase was concd The ppt was dissolved in deionized H_2O, then acidified to pH 2 0, and extracted with Et_2O Proteoglycan A was treated with aq 0 5 M NaOH at 60° for 90 min under a N_2 stream The alkaline soln was acidified to pH 2 0 and extracted with Et_2O Each solvent extract was subjected to TLC and developed with C_6H_6–dioxan–HOAc (90 25 4) A phenolic substance on TLC was detected by spraying with 2% $FeCl_3$ and diazotized sulphanilic acid

Isolation and identification of ferulic acid Proteoglycan A (20 g) was dissolved in 0 5 M NaOH (100 ml) and treated at 60° for 90 min in N_2 The hydrolysate was acidified to pH 2 and extracted with Et_2O The Et_2O extract was subjected to prep silicic acid (Mallinckrodt)-dry column chromatography (3 × 70 cm, M Woelm, DDC-5), eluted with C_6H_6–dioxan–HOAc (90 25 4) When the developing solvent reached the end of the column, the dry powder was extruded from the column and divided into 5 cm sections Each fraction was eluted with EtOH, and fractions which gave a positive phenolic reaction (diazo and $FeCl_3$ reactions) were subjected to prep TLC on silica gel H (C_6H_6–dioxan–HOAc, 90 25 4) The zone which gave the red-orange colour with the diazo reagent was eluted with EtOH, then the solvent was evapd under red pres The concentrate was suspended in Et_2O Evapn of the solvent at room temp afforded colourless needles, mp 167–168 5° These were identified as ferulic acid by direct comparison (TLC, UV, IR, GLC) with an authentic sample The hexane soln of TMSi-ether derivative [15] was resolved on a Yanagimoto Type G-8 gas chromatograph fitted a flame-ionization detector GLC separation was on an OV-101 on Chromosorb W-A-W (stainless steel column, 100 × 0 3 cm, programmed from 100 to 250° at 6°/min)

Isolation and identification of ferulic acid hydroxamate Proteoglycan A1 (100 mg) was treated with alkaline hydroxylamine (2 ml) at 40° for 90 min Hydroxylamine soln was prepared by adjusting the pH of 4 M hydroxylamine–HCl to 10 using NaOH [16] Hydroxylamine-treated proteoglycan A1 was subjected to Sephadex G-25, and the resulting hydroxamate and hydroxamate-free proteoglycan were detected using $FeCl_3$–HCl soln (5% $FeCl_3$, w/v in 3 M HCl) [17] and the phenol–H_2SO_4 method [18], respectively As hydroxylamine does not react with the $FeCl_3$ reagent, it was detected by the method of Seifter *et al* [16]

Proteoglycan A1 (5 g) was treated with alkaline hydroxylamine as described above, acidified to pH 2 0 and then extracted with EtOAc The EtOAc extract was concd, taken up in MeOH and subjected to Sephadex LH-20 The second peak on the Sephadex LH-20 profile was rechromatographed on Sephadex LH-20 and the major peak was concd Derivative UV and MS of the isolated and authentic hydroxamates were recorded on a Yanagimoto high order derivative UV–visible spectrophotometer and a JEOL JMS-01SG-2 high mass spectrometer, respectively

Synthesis of authentic ferulic acid hydroxamate The authentic hydroxamate was synthesized according to the modified method of Boschager [19] Ferulic acid Me ester was synthesized using diazomethane Solns of KOH in MeOH were added with shaking to the soln of NH_2OH HCl in MeOH After complete precipitation of KCl in an ice bath, the acid Me ester was added and the mixture filtered immediately, and the filtrate concd The K salt was suspended in 1 25 M HOAc, stirred and heated until a clear soln was obtained The soln was cooled to room temp and concd The concentrate was dissolved in MeOH–EtOAc, when the hydroxamate crystallized at −5° The synthetic hydroxamate, recrystallized at MeOH|EtOAc (3 ×), had molecular formula $C_{10}H_{11}NO_4$ (Found C, 57 49, H, 5 44, N, 6 7 Calc C, 57 39, H, 5 34, N, 6 69%) on the basis of elemental analysis Its physicochemical properties were measured by NMR and IR, and it was determined as ferulic acid hydroxamate

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