EVIDENCE FOR NOVEL LINKAGES IN A GLYCOPROTEIN INVOLVING β-HYDROXYPHENYLALANINE AND β-HYDROXYTYROSINE

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Summary: Cutinase, an extracellular enzyme from *Fusarium solani f. pisi* contains about 4% covalently attached carbohydrates. Treatment of the enzyme with alkali resulted in β-elimination and generation of dehydroamino acids absorbing at 241 nm. NaB^3H_4 treatment in 0.1 N KOH followed by hydrolysis of the labeled protein gave rise to tritiated alanine, α-aminobutyric acid, phenylalanine, and tyrosine. Chemical and enzymatic degradation of the labeled phenylalanine showed that this amino acid was a 1:1 mixture of D- and L-stereoisomers and that ^3H was equally distributed between the α- and β-positions. Therefore it is concluded that this glycoprotein contained O-glycosidic linkages not only at serine and threonine residues but also at β-hydroxyphenylalanine and β-hydroxytyrosine; the latter two have not been found heretofore.

INTRODUCTION

Cutinase, an extracellular enzyme produced by plant pathogenic fungi, is presumed to play a role in the entry of pathogens into plants by digesting the insoluble polyester, cutin, which is the structural component of plant cuticle. This enzyme has been isolated in homogeneous form from *Fusarium solani pisi* (1) and *Fusarium roseum culmorum* (2). Our attempts to determine the primary structure of this new "active serine"-containing hydrolase revealed that this enzyme is a glycoprotein containing about 4% carbohydrate (unpublished results). In this paper we present evidence that β-hydroxyphenylalanine and β-hydroxytyrosine, in addition to serine and threonine, are involved in the glycosidic linkage between the carbohydrate and this protein. Such linkages between proteins and carbohydrates have not been detected heretofore.

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EXPERIMENTAL

Materials. Sodium boro[^{3}H]hydride (293 mCi/mmole) was purchased from Amersham/Searle Corp. Phenylalanine ammonia lyase isolated from *Rhodotorula glutinis* (P-L Biochemicals) was used after gel filtration through a sepharose column. D-amino acid oxidase (hog kidney) and L-amino acid oxidase (snake venom) were purchased from Sigma Chemical Co. Cutinase I (*F. pisi*) was prepared as described before (1). Protein was determined by the method of Lowry et al. (3). ^{3}H was assayed by liquid scintillation spectrometry with Aquasol as the counting fluid.

Alkaline NaB^3H_4 Treatment of Cutinase. Treatment of the protein with alkaline NaB^3H_4 was carried out by a procedure similar to that described by Tanaka and Pigman (4). About 10 mg of electrophoretically homogeneous cutinase I was treated with 0.1 N KOH containing 0.2 M NaB^3H_4 (18 mCi) for 216 hr at 4°C in the dark. Then 0.2 ml of 0.08 M $PdCl_2$ and one drop of n-octanal were added and the reaction mixture was incubated for 2 hr at 22°C. The final mixture was acidified with acetic acid and lyophilized. The acidification and lyophilization were repeated several times until the mixture contained negligible amounts of exchangeable tritium. The mixture was dissolved in 1 ml of H_2O and centrifuged. The supernatant was subjected to gel filtration using a Bio-Gel P-2 column (1.2 × 110 cm) with H_2O as the eluant. The tritiated protein fraction was lyophilized and hydrolyzed in 6 N HCl for 24 hr and any exchangeable ^{3}H was removed by repeated lyophilization. Amino acids were separated with a polystyrene-sulfonate column (AA15, Beckman) of Beckman-Spinco Amino Acid Analyzer. The labeled amino acids were de-salted using Dowex 50W-X8 (50-100 mesh) in NH_4^+ form. The identity of these amino acids was confirmed by thin-layer chromatography on Silica Gel-G (0.5 mm) plates with n-propanol:34% NH_4OH (7:3 v/v) as the developing solvent. After development the silica gel scraped from the various areas of the chromatogram was assayed for ^{3}H directly by liquid scintillation spectrometry.

Enzymatic Deamination of Phenylalanine and Tyrosine. Phenylalanine was incubated with 0.5 mg of L-phenylalanine ammonia lyase in pH 8.7 borate (Na^+) buffer (33 mM) at 30°C for 3 hr. The mixture was acidified and extracted 4 times with ethyl ether. The combined ether extract was washed twice with water and the ether layer was then extracted 4 times with diluted NH_4OH; the combined NH_4OH extract was lyophilized. The residue was dissolved in a minimal volume of dilute NH_4OH and chromatographed on silica gel with n-propanol:34% NH_4OH (9:1) as the developing solvent. The labeled tyrosine was treated with the ammonia lyase and the products were analyzed in an identical manner. The unreacted amino acids were well separated from the deaminated products in both cases.

Chemical Degradation of Phenylalanine and Cinnamic Acid Derived from it. The permanganate-periodate oxidation of cinnamic acid derived from labeled phenylalanine was done by the procedure described by Von Rudloff (5). The reaction mixture was vigorously stirred for 3 hr at room temperature and acidified with 1 N HCl followed by the addition of sodium metabisulfite to decolorize the mixture. The solution was repeatedly extracted with ethyl ether. The ether extract was washed with water followed by dilute NH_4OH extraction. The ammonium benzoate was subjected to thin-layer chromatography on Silica Gel-G using a 9:1 mixture of n-propanol and 34% NH_4OH. The method of Gilvarg and Bloch (6) as modified by Hanson and Havir (7) was used for the chemical degradation of phenylalanine. Labeled phenylalanine was dissolved in 0.74 ml of H_2O containing 6 mg of $KMnO_4$, and 60 µl of H_3PO_4. The solution was heated at 95°C for 40 min, cooled, and the remaining $KMnO_4$ reduced with form-

aldehyde. The exchangeable ^3H was removed by sublimation. Samples of the sublimate were taken for ^3H assays. The residue was dissolved in dilute NH_4OH and chromatographed on silica gel thin-layer plates.

Determination of the Stereoisomer Composition of Phenylalanine. The labeled phenylalanine was incubated with D-amino oxidase or L-amino acid oxidase. For D-amino acid oxidase, 16 mM sodium pyrophosphate, pH 8.3, was used whereas for L-amino acid oxidase, 66 mM Tris buffer, pH 7.8, containing 0.047 M KCl was used. All the reaction mixtures contained 10 µg catalase and 100 µg amino acid oxidase in a final volume of 1 ml. The reaction was carried out at 30°C for 3 hr and the reaction was terminated by adding 0.2 ml of 1 N HCl. The mixture was loaded on Dowex 50W column and the resin was first washed with H_2O to remove the reaction products. The unreacted phenylalanine was recovered by elution with 2 N NH_4OH, lyophilized, and divided into two portions. One portion was treated with the same amino acid oxidase and the other with the oxidase of the opposite stereospecificity, and in each case products were analyzed as described above.

RESULTS AND DISCUSSION

Classical colorimetric tests for carbohydrates (8) showed that the electrophoretically homogeneous cutinase I from *F. solani pisi* contained 3.5 to 4.5% carbohydrates, and SDS disc gel electrophoresis showed that the carbohydrate was covalently attached to the protein. In order to test for the presence of O-glycosidic linkages at serine and threonine, which are present in cutinase I in substantial quantities (1), the enzyme was treated with alkali and the absorbance at 241 nm was measured, because the dehydroamino acids, which would be generated by β-elimination, absorb strongly at this wave length (9). As shown in Fig. 1, absorbance at 241 nm increased sharply upon treatment of cutinase with 0.4 N KOH at 30°C, while free threonine and serine did not show any increase in absorbance at 241 nm when similarly treated with KOH. These results strongly suggest that serine and threonine and possibly other residues in the protein have O-glycosidically linked carbohydrate.

In order to determine the nature of the amino acid residues involved in the β-elimination, cutinase was treated with 0.1 N KOH and 0.2 M NaB^3H_4 at 4°C for 216 hr. When the products were subjected to gel filtration with Biogel P-2, the protein, which was eluted at the void volume, and the carbohydrates, which were eluted later, contained ^3H. Upon hydrolysis of the recovered labeled protein with 6 N HCl for 24 hr, virtually all of the ^3H

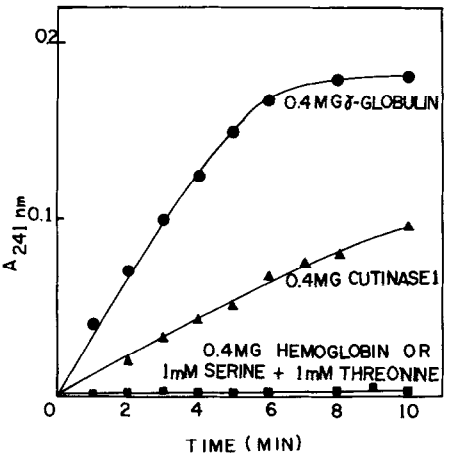

Figure 1. Changes in absorbance upon treatment of proteins with 0.4 N KOH at 30°C.

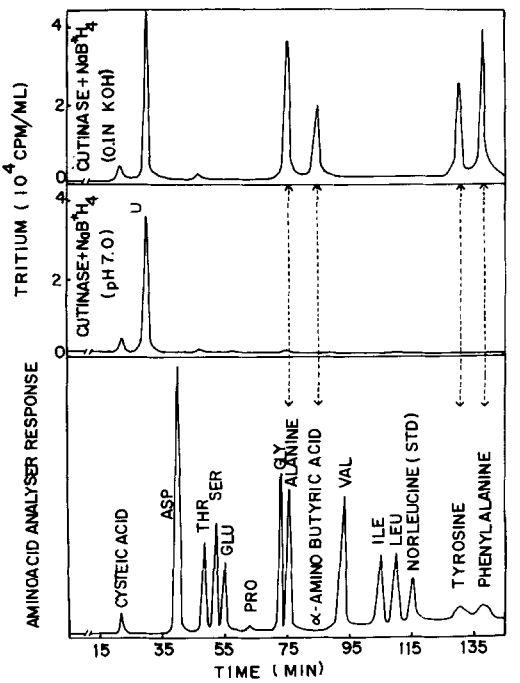

Figure 2. Distribution of radioactivity among the amino acids in the hydrolysate of the protein isolated by Bio-gel P-2 gel filtration of NaB^3H_4 treated cutinase. The bottom tracing shows an amino acid analyzer pattern obtained from a hydrolysate of untreated cutinase. The conditions of NaB^3H_4 treatment are indicated in the appropriate tracing and are described in the text.

contained in the protein was recovered in the hydrolysis products with little ^3H in the water. When the hydrolysis products were subjected to ion exchange chromatography in an amino acid analyzer, five major labeled products were found (Fig. 2). Two of these coincided with alanine and α-aminobutyric acid, which are expected to be formed by the NaB^3H_4 reduction of the α-aminoacrylic acid, and α-aminocrotonic acid, which would be generated by β-elimination of carbohydrates attached to serine and threonine residues by O-glycosidic bonds. These two labeled amino acids were further identified by thin-layer chromatography as described under the Experimental section. The labeled component U, which was eluted immediately after cysteic acid from the amino acid analyzer, was tentatively identified as L-gulonic acid; further information concerning this component will be reported in a later publication.

In addition to the three labeled components discussed above, two other labeled compounds were eluted from the amino acid analyzer, and these emerged with phenylalanine and tyrosine from the column. Thin-layer chromatography with authentic standards further showed that the radioactivity in these two components was contained exclusively in phenylalanine and tyrosine (Fig. 3). Treatment of the labeled phenylalanine and tyrosine fractions with

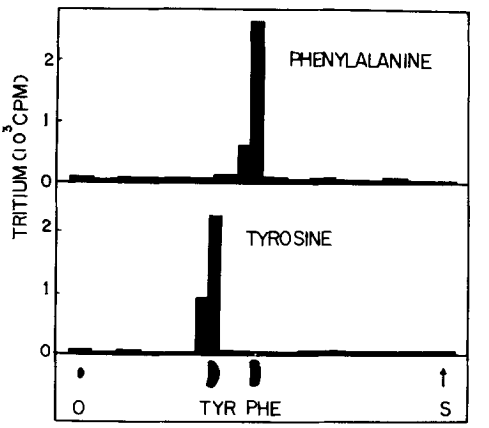

Figure 3. Distribution of ^3H in the thin-layer chromatograms of the phenylalanine and tyrosine fractions obtained from the amino acid analysis shown in Fig. 2 (top tracing); O, origin; S, solvent front.

phenylalanine ammonia lyase of *R. glutinis*, which is known to deaminate both phenylalanine and tyrosine, gave rise to labeled cinnamic acid and labeled p-hydroxycinnamic acid, respectively. These two products were identified by thin-layer chromatography as described in the Experimental section. These results showed that labeled phenylalanine and tyrosine were in fact produced by the treatment of cutinase with alkaline NaB^3H_4. That these amino acids did not naturally occur in the protein as the dehydroderivatives was shown by the observation that phenylalanine and tyrosine, as well as alanine and α-aminobutyric acid, were not labeled by NaB^3H_4 when the treatment was done without the alkaline β-elimination (Fig. 2). Thus, the above results strongly suggest that in this glycoprotein β-hydroxyphenylalanine and β-hydroxytyrosine, in addition to serine and threonine, are linked to carbohydrates by O-glycosidic bonds.

If the above conclusion is correct, ^3H in these amino acids should be located exclusively at the α- and β-carbons (Fig. 4). In order to determine the location of the ^3H in phenylalanine, the labeled amino acid was oxidized with $KMnO_4$ in H_3PO_4 and nearly equal amounts of ^3H were found in water and in phenylacetic acid produced by the oxidation (Table 1). Thus, approximately one-half of the ^3H was at the α-position. Benzoic acid produced from phenylalanine, as shown in Fig. 4, contained no ^3H, proving that all of the ^3H contained in phenylalanine was at the α- and β-carbons. These results show that in the labeled phenylalanine, ^3H was distributed equally between α- and β-positions.

If the dehydrophenylalanine generated by β-elimination was not in a sterically hindered environment in the protein, the NaB^3H_4 reduction should result in a D,L-mixture of phenylalanine. In fact, sequencial treatment of the tritiated phenylalanine with L-amino acid oxidase and D-amino acid oxidase, followed by analysis of the distribution of ^3H in the products (Table 1) showed that this labeled amino acid consists of a 1:1 mixture of D- and L-stereoisomers.

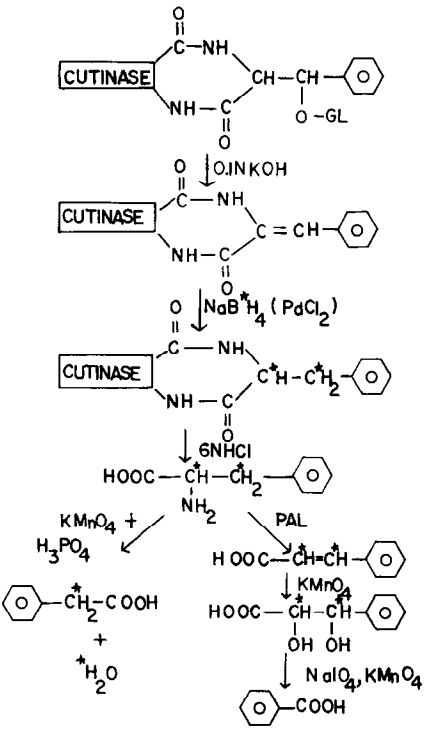

Figure 4. Schematic representation of the attachment of a carbohydrate (GL) to cutinase via an O-glycosidic linkage at a β-hydroxyphenylalanine residue and the degradation reactions used to deduce the structure. * represents ^{3}H.

The results discussed above show that $NaB^{3}H_4$ treatment of cutinase after β-elimination gave rise to four labeled amino acids which were identified as alanine, α-aminobutyric acid, phenylalanine, and tyrosine. The chemical and enzymatic degradation of the labeled phenylalanine showed that it was a D,L-mixture and that ^{3}H was equally distributed between α- and β-carbons, and presumably a similar distribution of ^{3}H is present in the other three amino acids. Therefore, it is concluded that in this glycoprotein, carbohydrates are attached by O-glycosidic linkages to serine, threonine, β-hydroxyphenylalanine, and β-hydroxytyrosine. The former two amino acids have been known to be involved in such linkages in other glycoproteins (10), but the latter two amino acids have not been heretofore found to be involved in the attachment of carbohydrates to proteins.

Table 1. Release of ^3H into water by amino acid oxidases from [^3H]-phenylalanine derived from KOH-NaB$^3H_4$ treated cutinase.

Treatment	Radioactivity in Water	Radioactivity in Unreacted Phenylalanine
	(%)	
Experiment 1		
D-Amino acid oxidase	43	57
D-Amino acid oxidase followed by L-Amino acid oxidase	90	10
D-Amino acid oxidase followed by D-Amino acid oxidase	50	50
L-Amino acid oxidase	42	58
L-Amino acid oxidase followed by D-Amino acid oxidase	90	10
L-Amino acid oxidase followed by L-Amino acid oxidase	51	49
Experiment 2		
$KMnO_4$ + H_3PO_4 (95°C)	55	45*

*Phenylacetic acid. Experimental conditions are described in the text.

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