Phytochemistry, Vol. 26, No. 6, pp. 1577–1581, 1987.
Printed in Great Britain.

0031-9422/87 $3.00 + 0.00

PURIFICATION AND CHARACTERIZATION OF CHLOROGENIC ACID: CHLOROGENATE CAFFEOYL TRANSFERASE IN SWEET POTATO ROOTS

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(*Received* 21 *October* 1986)

Key Word Index—*Ipomoea batatas*; Convolvulaceae; sweet potato; chlorogenic acid:chlorogenate caffeoyl transferase; enzyme purification and characterization; phenylpropanoid; chlorogenic acid; isochlorogenic acid.

Abstract—A chlorogenic acid:chlorogenate caffeoyl transferase, which catalyses the conversion of chlorogenic acid to isochlorogenic acid, has been purified 3600-fold from sweet potato root and characterized. The purified enzyme yielded one band on polyacrylamide gel electrophoresis both under non-denaturing and denaturing conditions. The enzyme was shown to consist of a single polypeptide of molecular weight 25 000 by gel filtration chromatography and SDS-gel electrophoresis. pI of the enzyme was 4.6. The optimum pH of the enzyme reaction was 5.0 (50% at pH 3.7 and 6.8). The enzyme did not have any cofactor requirements. The enzyme showed a strict substrate specificity toward chlorogenic acid, for which the K_m value was 0.87 mM. The enzyme activity changed in a manner indicating its involvement in the conversion of chlorogenic into isochlorogenic acid during incubation of sliced sweet potato root tissues.

INTRODUCTION

Chlorogenic acid (3-*O*-caffeoylquinic acid) and isochlorogenic acid (3,5-di-*O*-caffeoylquinic acid) are ubiquitous polyphenols in the plant kingdom [1–4]. When sweet potato root tissue is infected or wounded, the synthesis of chlorogenic acid and isochlorogenic acid is increased [5–7]. Using wounded sweet potato roots, we have studied the biosynthetic pathway of these phenolic compounds [8–13]. Our earlier tracer experiment indicated that chlorogenic acid is rapidly metabolized into isochlorogenic acid in sweet potato root [8]. Furthermore, since the specific radioactivities of chlorogenic acid and isochlorogenic acid were almost the same in sweet potato root discs supplied with D-[G-^{3}H]quinic acid [10], the conversion of chlorogenic acid into isochlorogenic acid may occur in a one step reaction *in vivo*.

In a preceding paper [12], we detected the enzyme in sweet potato root which produced isochlorogenic acid from chlorogenic acid by the reaction shown in Fig. 1. In the enzyme reaction, chlorogenic acid acted both as acyl donor and acceptor molecule. The present paper deals with purification and characterization of this enzyme, chlorogenic acid:chlorogenate caffeoyl transferase. Furthermore, we present evidence for the involvement of the enzyme in isochlorogenic acid biosynthesis in sweet potato root.

RESULTS

Purification of chlorogenic acid:chlorogenate caffeoyl transferase

Sweet potato slices which had been incubated at 25° for 2 days to induce the enzyme were used as the source of the enzyme. An acetone powder of the slices was prepared which removed polyphenols which could interfere with the enzyme assay. The enzyme was extracted from the powder and purified 3600-fold according to the scheme shown in Table 1: ammonium sulphate fractionation, followed by column chromatography on Sephadex G-25, DEAE-Toyopearl, DEAE-Sephacel, hydroxyapatite, Superose 12 and polyacrylamide gel electrophoresis.

Properties of the enzyme

The purified enzyme was quite stable and could be stored in ice for 1 month without appreciable loss of activity. The enzyme did not require the addition of any cofactor for activity.

The purified enzyme preparation showed one band on polyacrylamide gel electrophoresis under non-denaturing conditions, to which the enzyme activity was confined (data not shown). It showed also one band (*ca* 25 000) on SDS-polyacrylamide gel electrophoresis. The molecular

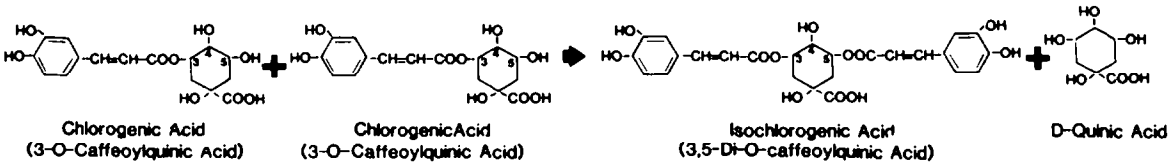

Fig. 1. Enzymatic reaction catalysed by chlorogenic acid:chlorogenate caffeoyl transferase

Table 1. Summary of the purification of chlorogenic acid:chlorogenate caffeoyl transferase from sweet potato roots

Purification step	Total protein (mg)	Total enzyme activity (units)	Specific enzyme activity (units/mg)	Purification (fold)	Yield (%)
Crude extract	2380	615	0.26	1	100
Ammonium sulphate	725	270	0.37	1.4	44
Sephadex G-25	470	170	0.36	1.4	28
DEAE-Toyopearl	200	240	1.20	4.6	29
DEAE-Sephacel	12	115	9.58	37	19
Hydroxyapatite	2.9	50	17	66	8
Superose 12	0.38	29	76	290	5
Polyacrylamide gel electrophoresis	0.015	14	930	3600	2

The enzyme was extracted from 1423 g of sweet potato root slices which had been incubated for 2 days and assayed using the standard method described in the Experimental.

weight of the enzyme was estimated to be 25 000 by gel filtration chromatography on Superose 12. Thus, the enzyme seems to be composed of a single polypeptide of M_r 25 000. The pI of the enzyme was determined to be 4.6 by gel electrofocusing (data not shown). The enzyme showed an optimum reaction rate at pH 5.0, (50% at pH 3.7 and 6.8, data not shown).

The substrate specificity of the enzyme was examined in terms of two types of activity; one was acyl-transferring activity between homologous compounds and the other was acyl-transferring activity between heterologous compounds (Table 2). A single substrate compound was included in the assay mixture to determine the former activity, while two substrate compounds (acyl donor and acceptor compounds) were included in the assay mixture to determine the latter activity. The enzyme showed a strict substrate specificity; it catalysed only the transfer of a caffeoyl moiety between two molecules of chlorogenic acid to produce isochlorogenic acid. The K_m value of the enzyme for chlorogenic acid was determined to be 0.87 mM by a Lineweaver–Burk plot (data not shown). The enzyme showed no affinity for hydroxycinnamoyl D-glucoses, *t*-cinnamoylquinic acid or *p*-coumaroylquinic acid. The enzyme was also incapable of catalysing transesterification between heterologous compounds; it neither transferred the caffeoyl moiety of chlorogenic acid to any heterologous compounds tested nor showed the activity of 1,2-diacyl-*sn*-glycerol:sterol acyl transferase.

Time course of change in enzyme activity and contents of chlorogenic acid and isochlorogenic acid in sweet potato root discs during incubation

The change in enzyme activity was monitored as well as the change in the amount of chlorogenic and isochlorogenic acid in sweet potato root discs during incubation (Fig. 2). The enzyme activity changed in a way suggesting that the enzyme is involved in the synthesis of isochlorogenic acid from chlorogenic acid in sweet potato roots.

DISCUSSION

Sweet potato roots contain the enzyme, hydroxycinnamoyl D-glucose:quinate hydroxycinnamoyl transferase, which produces chlorogenic acid by transesterification between caffeoyl D-glucose and D-quinic acid [13]. Chlorogenic acid is then converted into isochlorogenic acid, in which two caffeoyl moieties are conjugated with one D-quinic acid moiety through ester bonds. Therefore, we anticipated that the second caffeoyl moiety in the isochlorogenic acid molecule might be introduced by a similar transesterification reaction with another caffeoyl D-glucose. However, this was not the case. The second

Table 2. Substrate specificity of chlorogenic acid:chlorogenate caffeoyl transferase

Substrate	Activity (nmol/min/mg protein)
3-*O*-Caffeoylquinic acid (chlorogenic acid)	2.8
p-Coumaroylquinic acid*	0
t-Cinnamoylquinic acid*	0
Caffeoyl D-glucose*	0
p-Coumaroyl D-glucose*	0
t-Cinnamoyl D-glucose*	0
Shikimic acid†	0
L-Malic acid†	0
meso-Tartaric acid†	0
myo-Inositol†	0
Glycerol†	0
D-Glucose†	0
Diacylglycerol (acyl donor), cholesterol (acceptor)‡	0

Assays were carried out by the method described in the Experimental. The final concentrations of all substrates marked by * or † were 5.0 mM.

*Assayed in the assay mixture containing a single substrate.

†Assayed in the assay mixture containing chlorogenic acid (5 mM) as acyl donor. All assay mixtures after incubation contained isochlorogenic acid, 3,5-di-*O*-caffeoylquinic acid, produced by the acyl-transfer between chlorogenic acid molecules themselves.

‡Assayed by the method in ref. [23]. The final concentrations of diacylglycerol, phosphatidylcholine and [7-α-^{3}H]cholesterol were 0.05, 0.04 and 5 mM, respectively.

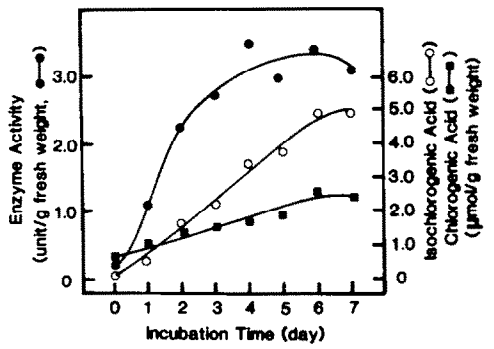

Fig. 2. Time course of change in enzyme activity and contents of chlorogenic acid and isochlorogenic acid in sweet potato root discs during incubation. The experimental conditions are given in the Experimental.

ester bond in isochlorogenic acid was formed by a different mechanism with a different enzyme; the caffeoyl moiety of chlorogenic acid was from caffeoyl D-glucose, while the second caffeoyl moiety of isochlorogenic acid was from another chlorogenic acid molecule. The two enzymes responsible for the respective reactions, hydroxycinnamoyl D-glucose:quinate hydroxycinnamoyl transferase and chlorogenic acid:chlorogenate caffeoyl transferase, were two different enzyme proteins. The pI of the former enzyme was 8.5 [13], while that of the latter was 4.6.

Analogous enzyme reactions have been reported in other secondary plant metabolism. Thus, Gross found an enzyme in oak leaves which forms digalloylglucose, utilizing β-glucogallin both as acyl donor and acceptor molecule [14]. Dahlbender and Strack also reported a similar enzyme in the cotyledons of *Raphanus sativus* seedlings grown in the dark [15]. This enzyme catalyses the transfer of the sinapoyl moiety of 1-*O*-sinapoyl D-glucose to the C-2 hydroxy group of the glucose moiety of another molecule of 1-*O*-sinapoyl D-glucose to form 1,2-di-*O*-sinapoyl D-glucose. Both enzymes are involved in the transfer of the acyl group between two molecules of the glucose ester of an aromatic carboxylic acid. On the other hand, the enzyme isolated from sweet potato roots in the present paper did not show any activity toward glucose esters of hydroxycinnamic acids such as *t*-cinnamoyl D-glucose, *p*-coumaroyl D-glucose and caffeoyl D-glucose (Table 2).

The enzymes involved in secondary metabolism generally show broad substrate specificity. Indeed, UDPG:*t*-cinnamate glucosyl transferase and hydroxycinnamoyl:quinate hydroxycinnamoyl transferase which are involved in chlorogenic acid biosynthesis in sweet potato roots showed a rather broad substrate specificity [11, 13]. Therefore, the strict substrate specificity of chlorogenic acid:chlorogenate caffeoyl transferase seems to be a unique characteristic. We could not detect any enzyme reaction product with any of the substrates tested except chlorogenic acid using the assay method described in the Experimental. It should be mentioned, however, that non-radioactive compounds were used as substrates in these assays except in the case of 1,2-diacyl-*sn*-glycerol:sterol acyl transferase activity. The assay method was sensitive enough to detect the enzyme reaction product of chlorogenic acid, but might not be sensitive enough to detect trace amounts of reaction products from other substrates.

We have not examined the cellular distribution of chlorogenic acid and isochlorogenic acid in sweet potato roots. However, it is widely accepted that the secondary metabolites such as these are localized within the cell vacuole [16, 17]. Therefore, it seems reasonable to assume that chlorogenic acid:chlorogenate caffeoyl transferase is localized within the vacuole or tonoplast of sweet potato root cells. In support of this hypothesis, the optimum pH of the enzyme reaction was 5.0 and it is reported that the pH inside the vacuole is acidic and that vacuolar catabolic activity is greatest at an acidic pH [18, 19].

EXPERIMENTAL

Materials. Sweet potato (*Ipomoea batatas* Lam. cv Norin 1) roots were harvested in October and stored at 13° until used. The following materials were obtained from commercial sources: Sephadex G-25, DEAE-Sephacel and pre-packed column of Superose 12 (HR 10/30) (Pharmacia); DEAE-Toyopearl 650 M (TSK-gel, polyvinyl alcohol gel, Toyosoda); hydroxyapatite (Wako); chromatography paper (Whatman 3 MM); Ampholine (LKB, pH 4–6); Centricon 10 (Amicon); protein calibration kit (Boehringer Mannheim, size 2); electrophoresis calibration kit (Pharmacia); protein assay kit (Bio-Rad), [7-α-^{3}H]cholesterol (Amersham, 5.7 Ci/mmol).

Preparation of substrates. *t*-Cinnamoyl D-glucose was extracted from leaves of *Spirea thunbergii* Sieb. by the method of Tanabe and Kita [20] and identified by NMR. 1-*O*-*p*-Coumaroyl D-glucose and 1-*O*-caffeoyl D-glucose were chemically synthesized and identified by NMR as reported previously [11, 13]. 1-*O*-*t*-Cinnamoylquinic acid and 1-*O*-*p*-coumaroylquinic acid were enzymatically synthesized from 1-*O*-*t*-cinnamoyl D-glucose and 1-*O*-*p*-coumaroyl D-glucose, respectively, using hydroxycinnamoyl D-glucose:quinate hydroxycinnamoyl transferase prepared from sweet potato roots. Other substrates were obtained from commercial sources.

Enzyme assay. A reaction mixture consisting of 3.0 μmol chlorogenic acid, 0.1 μmol $MgCl_2$, 20 μmol acetate (pH 5.0) and the enzyme soln in a final vol. of 550 μl was incubated at 30° for 30 min. The reaction was terminated by adding 3 ml EtOH and 2 ml Me_2CO_2. The mixture was left at room temp. for 45 min and centrifuged at 2×10^3 *g* for 5 min. The supernatant was concd and applied to PC. The papers were developed with 5% HOAc. A UV-fluorescent band at R_f 0.23 (isochlorogenic acid) was cut out and eluted with a mixture of EtOH and H_2O (1:1)). Absorbance of the eluate at 324 nm was measured to calculate the amount of isochlorogenic acid produced using molar absorption coefficient (37 200 M^{-1} cm^{-1}). $MgCl_2$ was included in the reaction mixture for efficient precipitation of proteins and inorganic salts upon the addition of EtOH and Me_2CO_2 after enzyme reaction. One unit of activity was defined as the amount of enzyme which produced 1 nmol isochlorogenic acid/min under the assay conditions. When the crude enzyme preparation contaminated with polyphenoloxidase was assayed, a reaction mixture was incubated under N_2 gas to inhibit the oxidation of substrate and product.

Protein determination. Protein was determined by the method of Bradford [21]. Bovine serum albumin was used as standard.

Purification of the enzyme. All operations were carried out at 4°. Sweet potato slices (5 mm thick) which had been incubated at 25° for 2 days to induce the enzyme were used as the enzyme source. Acetone powder was prepared from these slices and the powder was extracted with K-Pi buffer, pH 7.0 (buffer A). The extract was fractionated with $(NH_4)_2SO_4$ and the ppt between 40% and 70%

satn was collected by centrifugation at $3.2 \times 10^4 g$ for 20 min. The ppt was dissolved in 25 mM K-Pi buffer, pH 7.5 (buffer B) and applied to a Sephadex G-25 column. The void vol. fraction from the column was collected and applied to a column of DEAE-Toyopearl which was washed with buffer B and buffer B containing 150 mM NaCl and then eluted with a linear gradient of NaCl between 150 and 400 mM in buffer B. The active fractions which eluted at 200–270 mM NaCl were pooled and precipitated with 70% $(NH_4)_2SO_4$ satn. The ppt was dissolved in buffer B and desalted with Sephadex G-25. The desalted soln was applied to a DEAE-Sephacel column which was washed with buffer B and buffer B containing 0.2 M NaCl, then eluted with a linear gradient of NaCl between 0.2 and 0.4 M. The active fractions eluted at 280–380 mM NaCl were pooled and precipitated at 85% $(NH_4)_2SO_4$ satn. The ppt was dissolved in pH 6.8, 10 mM K-Pi buffer (buffer C), then desalted using Sephadex G-25. The desalted soln was applied to a column of hydroxyapatite. The fractions with the enzyme activity were collected and condensed with Centricon 10. The condensed soln was applied to a Superose 12 pre-equilibrated with 50 mM phosphate buffer, pH 7.0 (buffer D) and eluted with the same buffer. The active fraction was collected and condensed with Centricon 10. The condensed sample was subjected to polyacrylamide gel electrophoresis under the conditions described below. A strip was cut from the edge of the slab after electrophoresis. The strip was stained with Coomassie Brilliant Blue (CBB) dye to localize protein. Five bands appeared on the stained gel. Each protein band was electro-eluted from the remaining unstained gel and assayed for enzyme activity. The enzyme activity was detected only with the 4th band from the top of gel. The active enzyme soln was condensed with Centricon 10 and was used for polyacrylamide gel electrophoresis under both denaturing (SDS) and non-denaturing conditions.

Polyacrylamide gel electrophoresis. The enzyme preparation was separated in a 1 mm thick slab consisting of 4% stacking gel and 10% running gel which contained 125 mM Tris–HCl buffer, pH 6.8 and 300 mM Tris–HCl buffer, pH 8.8, respectively. The gel was stained with CBB to detect the protein bands. To localize the enzyme protein, the bands on the unstained gel were electroeluted and assayed for enzyme activity.

SDS-polyacrylamide gel electrophoresis. The enzyme was denatured and reduced by boiling for 2 min in the loading buffer (5 mM Tris–HCl buffer, pH 6.8; 10% glycerol; 2% SDS; 2% 2-mercaptoethanol; 1×10^{-3}% bromphenol blue). The treated samples were separated in a 1 mm thick slab containing 4% acrylamide stacking gel and 10% running gel, with a buffer system according to Laemmli [22]. The gel was stained with CBB.

pI determination by gel electrofocusing. The enzyme was electrofocused on two gels of 5% acrylamide (0.5 × 7.5 cm) containing 2% Ampholine (pH 4–6) in 1400 volthours, using 10 mM glutamic acid as anode soln and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) as cathode soln. After electrofocusing, one gel was cut into 0.5-cm long discs, each of which was soaked in 0.5 ml H_2O at 30° for 5 hr on a shaker. The pH values of soaking soln were determined to monitor the pH gradient in the gel. Another gel was also cut into 0.5-cm long discs, each of which was soaked in 0.4 ml 0.2 M acetate buffer, pH 5.0, in ice for 15 hr on a shaker. The soaking soln was taken up and assayed for enzyme activity by the standard method.

Substrate specificity of the enzyme. The substrate specificity of the enzyme was examined in terms of two types of activities; one was acyl-transferring activity between homologous compounds and the other was acyl-transferring activity between heterologous compounds. A single substrate compound was included in the assay mixture to determine the former activity, while two substrate compounds were included in the assay mixture to determine the latter activity.

The various substrates (5 mM) were incubated in the standard assay mixtures and incubated at 30° for 60 min. In controls, the complete reaction mixtures were incubated with the boiled enzyme. The reaction was terminated by adding 3 ml EtOH and 2 ml Me_2CO_2. The mixture was left at room temp. 45 min and centrifuged at $2 \times 10^3 g$ for 5 min. The supernatant was concd and applied to TLC plates (AVICEL) which were run two dimensionally, in 5% HOAc and then *n*-BuOH–pyridine–H_2O, 14:3:3, (BPW). The developed plates were examined under long wave (302 nm) and short wave (260 nm) UV to detect any enzyme reaction products. A product was detected only with the reaction mixture containing chlorogenic acid. This product (3,5-dicaffeoylquinic acid) was scraped off, eluted with a mixture of EtOH and H_2O (1:1) and quantified as described above. When diacylglycerol (donor) and cholesterol (acceptor) were used as substrates, the enzyme was assayed by the reported method [23]. In a control experiment, spinach leaves were used as an enzyme source, which gave an expected amount of cholesterol ester in the standard assay system [23].

Determination of M_r *by gel filtration.* M_r of the enzyme was determined by gel filtration on Superose 12. The enzyme and cytochrome *c* (1300), chymotrypsinogen (25 000), chicken albumin (45 000), aldolase (14 700) and ferritin (54 000) in a protein calibration kit were eluted using buffer D, as elution buffer.

Time-course analysis of change in enzyme activity and contents of chlorogenic acid and isochlorogenic acid in sweet potato root discs during incubation. Discs (15 × 5 mm, 0.97 g) were prepared from sweet potato roots and incubated at 25° in a moist chamber. Twenty discs (19.4 g) were taken daily and homogenized in 30 ml chilled Me_2CO_2. The homogenate was filtered through paper and washed with 100 ml chilled Me_2CO_2. The filtrate and washings were combined and used for determination of contents of chlorogenic and isochlorogenic acids. The dried powder was extracted with 30 ml of buffer A containing 0.1% of 2-mercaptoethanol. The extract was assayed for enzyme activity by the standard method.

A known vol. of the Me_2CO_2 extract was run two-dimensionally on TLC (AVICEL) in 5% HOAc and BPW. The UV-fluorescent spots of chlorogenic and isochlorogenic acids were scraped off and eluted with a mixture of EtOH and H_2O (1:1). The absorbance of the eluate was read at 326 nm. The chlorogenic acid and isochlorogenic acid contents were calculated using the molar absorption coefficient, 186 000 and 373 000 $M^{-1} cm^{-1}$, respectively.

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