

# Metabolism of Ferulic Acid Sucrose Esters in Anthers of *Tulipa* cv. Apeldoorn: II. Highly Specific Degradation of the Esters by Different Esterase Activities

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Protein extracts from anthers of *Tulipa* cv. Apeldoorn catalyze the degradation of ferulic acid sucrose esters. Different products are generated when triferuloyl sucrose (TFS) and diferuloyl sucrose (DFS) were applied as substrates. By the aid of reversed-phase HPLC, TLC and spectroscopy the products could be identified as free ferulic acid, monoferuloyl sucrose ester [feruloyl-sucrose(mono)] and two different diesters of ferulic acid and sucrose [feruloylsucrose(di) and the endogenously occurring diferuloylsucrose (DFS)]. By means of protein fractionation (chromatofocusing, anion exchange HPLC and molecular sieving HPLC), four different enzyme activities involved in the degradation process could be separated. According to their catalytic properties, they were characterized as esterases (= EA).

The partially purified esterase activity I (EA I) obtained after fractionation by chromatofocusing catalyzes the formation of feruloylsucrose(di) and ferulic acid when TFS is used as substrate. Incubations with EA Ia or EA Ib isolated in smaller portions lead to the same product pattern.

The esterase activity II (EA II) degrades TFS to ferulic acid and DFS. DFS as substrate is only accepted by the EA I activities, in all three cases ferulic acid and feruloyl sucrose(mono) are formed as products. The kinds of different degradation reactions clearly indicate that one enzyme (= the EA II activity) catalyzes exclusively the formation of DFS from TFS.

Both enzymes, EA I and EA II, exhibit a high specificity towards ferulic acid sucrose esters. Hydroxycinnamic sucrose esters with only sinapic acid moieties do not function as substrates. When enzymatically formed sucrose esters like feruloylsucrose(di), feruloylsucrose(mono) and mono-sinapoylsucrose were used as substrates, no product formation could be observed. Applying SFS as substrate, only the ferulic acid moiety was released by EA I. Further, naturally occurring esters (glucose- and CoA-esters of *p*-coumaric, caffeic, ferulic and sinapic acid; chlorogenic acid; BGM) tested so far were not degraded by EA I and EA II. It is assumed that these esterase activities play a specific role in the ferulic acid metabolism in *Tulipa* anthers.

## Introduction

Esterases isolated from plant sources are often classified on the basis of their preferred substrates. Due to the various acyl moieties of artificial substrates, like  $\alpha$ -naphthylacetate and due to different inhibitors used, the esterases were characterized as

acyl esterases, carboxylesterases, arylesterases and acylesterases [1-5]. These enzymes are considered to be relatively nonspecific because natural substrates have not been defined. In most cases, the physiological functions of these esterases are obscure. As far as plant esterases are concerned, there are only a few reports upon the occurrence of esterases with a definite specificity towards natural endogenous substrates [6-9].

Previous studies on the metabolism of hydroxycinnamic acid conjugates in anthers of *Tulipa* cv. Apeldoorn have shown that protein preparations from anthers exhibit esterase activities hydrolyzing tri-[TFS] and diferuloylsucrose [DFS] [10]. These ferulic acid sucrose esters appear during the early stages of pollen differentiation and disappear during the later stages of pollen ripening. This "turn over" indicates that they are metabolically active compounds [11]. Therefore, the existence of highly specific esterases involved in the metabolism of DFS and TFS must be

**Abbreviations:** TFS, 6-feruloyl- $\alpha$ -*o*-glucopyranosyl-(1-2)- $\beta$ -*o*-(3',4'-diferuloyl)-fructofuranose; DFS, 6-feruloyl- $\alpha$ -*o*-glucopyranosyl-(1-2)- $\beta$ -*o*-(3'-feruloyl)-fructofuranose; DSS I, 6-sinapoyl- $\alpha$ -*o*-glucopyranosyl-(1-2)- $\beta$ -*o*-(3'-sinapoyl)-fructofuranose; DSS II,  $\beta$ -*o*-(1,3-disinapoyl)-fructofuranosyl- $\alpha$ -*o*-glucopyranoside; SFS,  $\beta$ -*o*-(1-sinapoyl-3-feruloyl)- $\alpha$ -*o*-glucopyranoside; TCA, trichloroacetic acid; HPLC, high performance liquid chromatography; TLC, thin layer chromatography; BGM, biochanin A 7-*o*-glucoside-6"-*o*-malonate; FGM, formononetin 7-*o*-glucoside-6"-*o*-malonate.

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postulated. Results presented in this paper verify previous studies on the occurrence of different enzyme activities hydrolyzing DFS and TFS, and demonstrate for the first time that these esterases exhibit an extremely high specificity towards sucrose esters with ferulic acid as acyl moiety.

## Material and Methods

### Plant material

Tulip bulbs (cv. Apeldoorn), purchased from Nebelung (Münster, F.R.G.), were cultivated in the Botanical Garden of the University of Münster. Anthers of early stages of pollen differentiation were used as enzyme source.

### Enzyme preparation

The procedure of enzyme preparation, the buffer composition and the methods of protein fractionation (high performance molecular sieving (Bio-Sil TSK), high performance anion exchange chromatography (Bio-Gel TSK), chromatofocusing (PBE 94) are described elsewhere [12]. As for molecular sieving, HPLC fractions of 0.5 ml were collected. HPLC-columns were achieved from Bio-Rad (München, F.R.G.).

### Substrates

To determine the substrate specificity the following naturally occurring hydroxycinnamic acid esters were used:

Sucrose esters: triferylolsucrose (TFS), diferylolsucrose (DFS), disinapoylsucrose I (DSS I), disinapoylsucrose II (DSS II), sinapoylferuloylsucrose (SFS) (for detail see abbreviations). DFS and TFS were isolated from anthers of *Tulipa* cv. Apeldoorn. DSS I from *Raphanus sativus* was a kind gift from Prof. Dr. Strack (Pharm. Biologie, Techn. Univ. Braunschweig). DSS II and SFS from *Polygala chamaebuxus* were kindly provided by Prof. Dr. Hostettmann, Lusanne, Switzerland.

Sinapoylsucrose(mono) [SS(mono)], feruloylsucrose(mono) [FS(mono)] and feruloylsucrose(di) [FS(di)] were produced enzymatically by *Tulipa* protein from SFS, DFS and TFS respectively.

### Glucose- and CoA-esters:

Feruloylglucose and *p*-coumaroylglucose were isolated from cell cultures of *Chenopodium rubrum*.

Caffeoylglucose and sinapoylglucose were enzymatically synthesized by a partially purified glycosyltransferase from anthers of *Tulipa* cv. Apeldoorn [12]. CoA-esters of *p*-coumaric, caffeic, ferulic and sinapic acid were synthesized chemically as described elsewhere [13].

BGM (biochanin A 7-*o*-glucoside-6''-*o*-malate) was a kind gift from Prof. Dr. Barz (University of Münster, F.R.G.), chlorogenic acid was acquired from Roth (Karlsruhe, F.R.G.).

When quantification of one of these esters was necessary by means of spectroscopy, the optical density of solutions containing the chromatographically purified product were measured at  $\zeta_{\max}$  and compared to the molar absorptivity of the corresponding free hydroxycinnamic acid (see Ref. [14]).

### Synthetic substrates:

$\alpha$ - and  $\beta$ -naphthylacetate were obtained from commercial sources (Sigma, Munich, F.R.G.).

### Enzyme assays and activity determination:

10 nmol of hydroxycinnamic acid ester in 10  $\mu$ l methanol (CoA-ester in 10  $\mu$ l 0.1 M potassium phosphate buffer pH 7.0) were added to 190  $\mu$ l of enzyme preparation.

The reaction was stopped after incubation at 35 °C for 180 min by addition of 10  $\mu$ l TCA. After centrifugation (8000  $\times$  g, 1 min), 150  $\mu$ l of the clear supernatant was applied to HPLC or extracted with ethylacetate for TLC-analyses. Enzyme activity was determined by HPLC measuring peak areas of the separated products.

Degradation of BGM,  $\alpha$ - and  $\beta$ -naphthylacetate was determined according to Hinderer *et al.* (1986) [9].

To test whether the products were formed enzymatically, heat-denaturated proteins (100 °C, 10 min) were used in control experiments.

### Chromatography

HPLC: Protein fractionation was carried out with a Bio-Rad system (Munich, F.R.G.).

For reversed phase HPLC, a chromatograph from Abimed (Düsseldorf, F.R.G.) was used incorporating a VIS/UV-detector and a computer integrator (CR 3A Shimadzu, Kyoto, Japan). Solvents for reversed phase HPLC: Solvent A: acetic acid (1% in



distilled water), solvent B: acetonitrile (85% in distilled water).

The column (250×4 mm) was packed with Nucleosil (18.5 µm; Macherey-Nagel, Düren; F.R.G.). Separations were accomplished by gradient elution with the following gradient profile (time, *t* in min): *t*<sub>0</sub> 10% B in A, *t*<sub>15</sub> 50% B in A, *t*<sub>20</sub> 50% B in A, *t*<sub>23</sub> to *t*<sub>25</sub> 100% B, *t*<sub>27</sub> 10% B in A, *t*<sub>45</sub> 10% B in A, flow rate 1 ml/min. The isolation of certain substances was achieved by HPLC using a chromatographic column (120×8 mm) packed with Lichrosorb RP-18/5 µm (Knauer, Bad Homburg, F.R.G.). The separations were accomplished by the same gradient described above, but with a flow of 2 ml/min.

#### TLC:

TLC was carried out as described elsewhere [10].

#### Identification of reaction products

The different products obtained after HPLC were collected separately and were analyzed with an Uvikon-810 spectrophotometer (Kontron, Düsseldorf, F.R.G.) to determine  $\zeta_{\max}$  and shift behaviour on alkaline and acid treatment. Products were re-chromatographed by TLC and characterized by their behaviour in UV-light (UV 350 nm) with and without NH<sub>3</sub> vapour. Identification was accomplished by co-chromatography with authentic reference substances (hydroxycinnamic acid ester and hydroxycinnamic acids). Reaction products were hydrolyzed with 1 N HCL for 30 min or 1 N NaOH for 5 min at 100 °C.

Hydrolysates were analyzed by HPLC (phenylpropanes) and TLC (sugars). Sugars were identified chromatographically by direct comparison with reference sugars or by the sucrose/glucose/fructose UV-test (Boehringer, Mannheim, F.R.G.). Sucrose was hydrolyzed as described in Ref. [15].

## Results

#### Identification of the reaction products formed by the esterase activities

When crude protein extracts of young anthers desalted by Sephadex G 25 were incubated with TFS and DFS as substrates, different products were found on HPLC diagrams (Fig. 1, A–E). The products, B, C and D were identified as *p*-coumaric acid, ferulic acid and DFS by Co-TLC and Co-HPLC with refer-

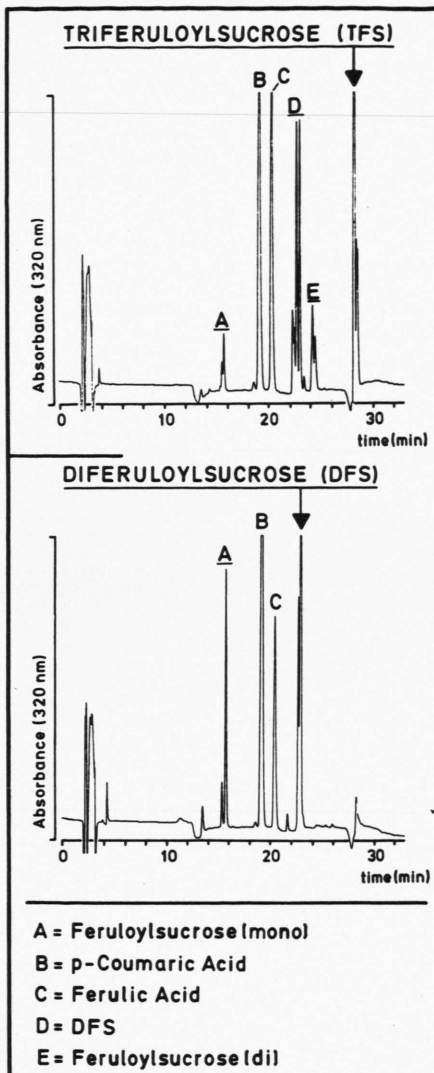


Fig. 1. HPLC-analyses of an enzyme assay with DFS (↓) and TFS (↓) as substrates and crude protein preparations.

ence substances by studying their typical behaviour under UV at 350 nm with and without NH<sub>3</sub> vapour, and by analyzing their spectroscopic data (Table I). Whenever DFS was produced in assays with TFS as substrate and a crude protein preparation, this ester appears in form of several peaks in the HPLC diagrams (Fig. 1, product D). Using a partial purified enzyme preparation obtained by chromatofocusing, DFS could be eluted as a single peak with a characteristic isomer (see Fig. 5). Obviously the crude en-

Table I. Chromatographic and spectroscopic properties of TFS, DFS and products enzymatically formed.

	$\lambda_{\max}$ [nm]			Behaviour in UV-light (350 nm)		Chromatographic properties		
	Methanol	Methanol + NaOH	Methanol + HCl	with NH <sub>3</sub> vapour	without NH <sub>3</sub> vapour	TLC ( $R_f \times 100$ ) SS I SS II	HPLC	
TFS	326	380	326	green	blue	10 90	see Fig. 1	
DFS	326	380	326	green	blue	30 80	see Fig. 1	
Product A FS(mono)	323	378	323	green	blue	65 50	see Fig. 1	
Product B <i>p</i> -coumaric acid	290	335	311	dark blue	dark blue	30 98	see Fig. 1	
Product C ferulic acid	316	347	323	blue	blue	25 95	see Fig. 1	
Product D DFS	326	380	326	green	blue	30 80	see Fig. 1	
Product E FS(di)	325	380	325	green	blue	35 75	see Fig. 1	

zyme extracts contain a non-specific enzyme activity with the ability to modify DFS. This assumption is confirmed by the fact that non-specific porcine liver esterase incubated with DFS leads to an identical peak pattern. Chemical acyl migrations, as observed by Birkofer *et al.* [16], can be excluded because DFS proved to be stable during incubation time.

Authentic reference substances were not available in order to identify the products A and E (see Fig. 1). With the exception of very slight differences in  $\zeta_{\max}$  all spectroscopic data and chromatographic properties of A and E are similar to those of DFS and TFS (Table I). Alkaline treatment of the products A and E resulted in the formation of ferulic acid and sucrose. This data ensures that these products formed enzymatically from DFS and TFS respectively are sucrose esters of ferulic acid. Comparing peak area values of A and E with those of free ferulic acid after HPLC of an assay with DFS or TFS and partially purified EA I, the following ratios could be obtained: ferulic acid:product A = 1:1 and ferulic acid:product E = 1:2. These ratios indicate that A represents a monoester and E a diester of ferulic acid; this assumption is confirmed by chromatographic data obtained by HPLC (Figs. 1, 4, 5) and TLC (results not shown). In order to determine the specific sites of esterification the two products were treated according to Linscheid *et al.* [15]. In both cases, by splitting sucrose we obtained only one product with ferulic acid as acyl moiety. As for product A, the hydroxycinnamic acid derivative resulting from sucrose splitting shows the chromato-

graphic behaviour of a monoderivative, while after hydrolyzing product E, a compound could be detected with retention values in TLC similar to a di-derivative. Based on all data available, the structure of A and E are proposed as shown in Fig. 3.

The formation of *p*-coumaric acid in assays with non-purified enzyme preparations is very remarkable (Fig. 1). To our knowledge up to now this product generated by an autocatalytic activity from an endogenous substrate of unknown high molecular structure which could be removed during protein fractionation from the – likewise – unknown *p*-coumaric acid producing enzyme activity (compare Fig. 1 with 4, 5). Studies to elucidate this phenomenon are underway.

#### *The separation of different esterase activities*

By using chromatofocusing, four esterase activities could be separated when TFS was applied as substrate (see Fig. 2). Three of them EAI, EAIa, EAIb; Fig. 2) produce additionally to DFS predominantly feruloylsucrose(di) from TFS, whereas a further esterase activity (EA II) catalyzes exclusively the formation of DFS from TFS. DFS is accepted as substrate only by the activities EA I, EA Ia and EA Ib.

When the protein fractions with the highest activities of EA I (fraction 20) and EA II (fraction 34, see Fig. 2) were subjected to anion exchange HPLC and molecular sieving HPLC the two enzymes could be separated only with difficulty. With the help of molecular sieving HPLC, a similar molecular weight

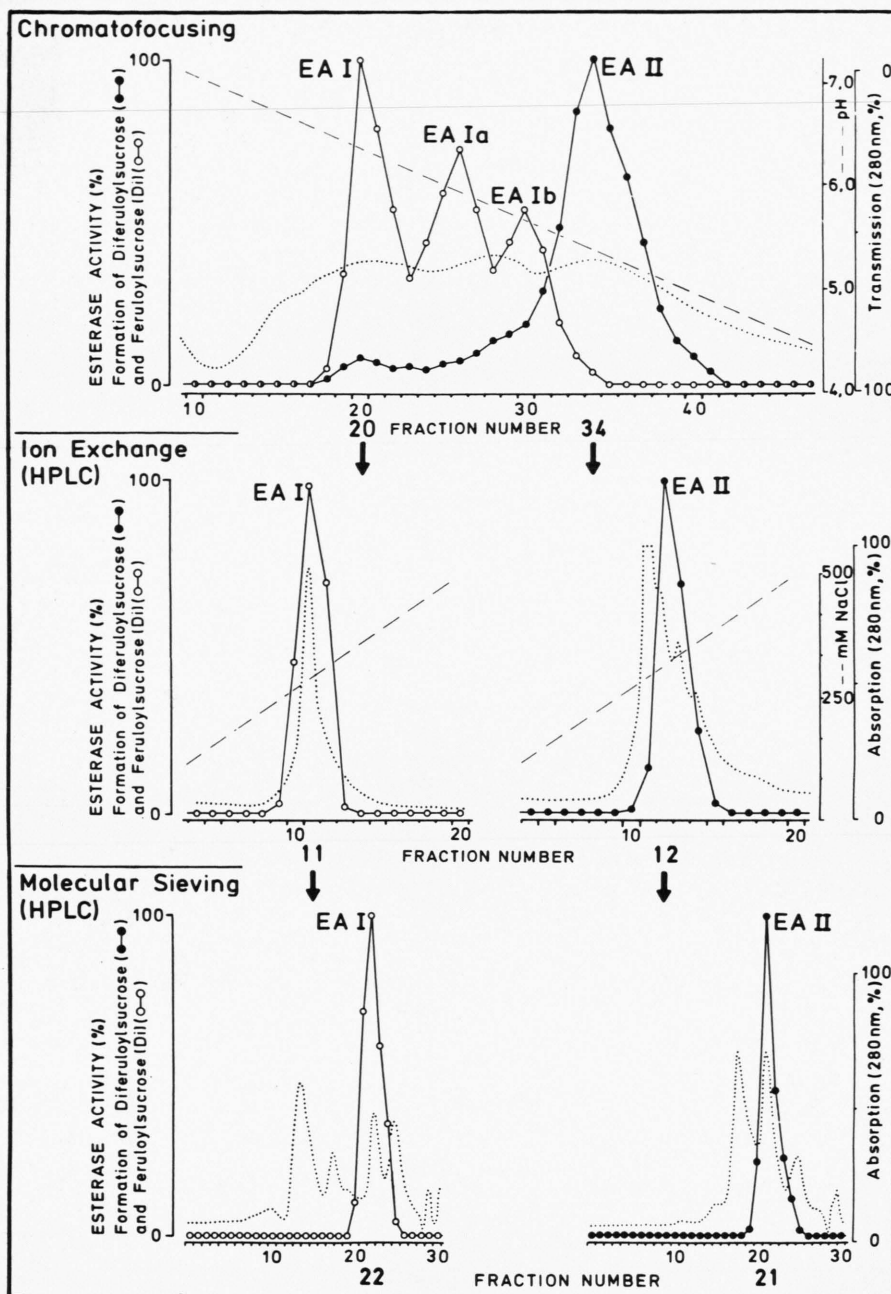


Fig. 2. Separation and chromatographic behaviour of the different esterase activities.

in the range of 45,000 D was found by comparison with reference proteins (see Ref. [12]).

#### *The substrate specificity of EA I and EA II*

A partially purified enzyme preparation obtained after chromatofocusing was used to elucidate the substrate specificity. EA I as well as EA II were

shown to be unable to hydrolyze diverse glucose- and CoA-esters of hydroxycinnamic acids, chlorogenic acid, BGM and  $\alpha$ - and  $\beta$ -naphthylacetate. The data summarized in Fig. 3 demonstrate the catalytic properties of EA I and EA II against natural sucrose esters available so far. Remarkable is the fact that EA II degrades exclusively TFS to DFS known as

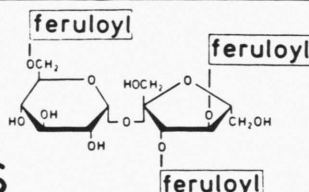
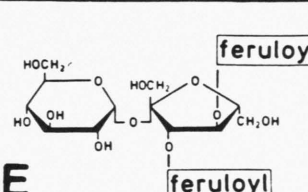
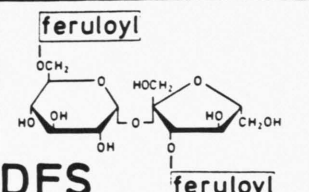
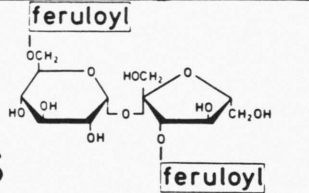
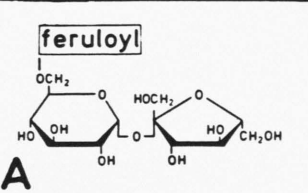
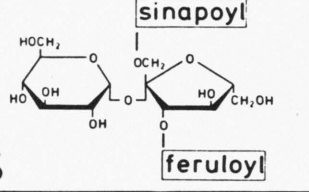
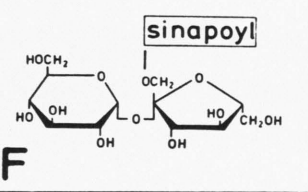
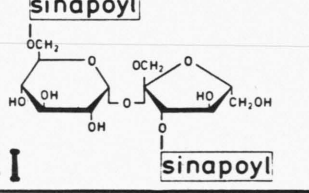
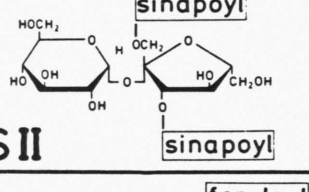
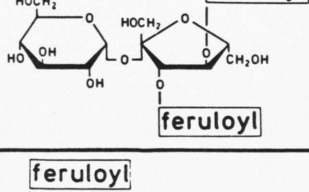
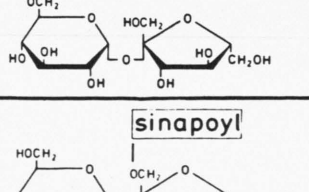
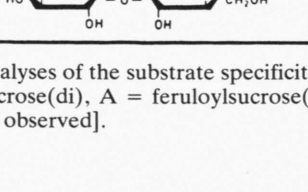
Substrates	EA I Products	EA II Products
 <p><b>TFS</b></p>	 <p><b>E</b></p>	 <p><b>DFS</b></p>
 <p><b>DFS</b></p>	 <p><b>A</b></p>	n.p.
 <p><b>SFS</b></p>	 <p><b>F</b></p>	n.p.
 <p><b>DSSI</b></p>	n.p.	n.p.
 <p><b>DSSI</b></p>	n.p.	n.p.
 <p><b>E</b></p>	n.p.	n.p.
 <p><b>A</b></p>	n.p.	n.p.
 <p><b>F</b></p>	n.p.	n.p.

Fig. 3. Analyses of the substrate specificity of EA I and EA II towards various hydroxycinnamic acid sucrose esters. [E = feruloylsucrose(di), A = feruloylsucrose(mono), see Fig. 1; F = sinapylsucrose(mono), see Fig. 4; n.p. = no product formation observed].



an endogenously occurring product. Other sucrose esters tested do not function as substrates. EA I hydrolyzes the genuine plant esters TFS and DFS, respectively and SFS (Fig. 3). In the case of SFS only ferulic acid was detectable as an enzymatically formed hydroxycinnamic acid, so that the monoester generated as the second product must represent the monosinapoylsucrose (Figs. 2, 3, F). Analogous to these arguments for product A generated after degradation of DFS by EA I (Fig. 4), a structure is proposed as shown in Fig. 3.

TFS hydrolyzed by EA I leads to the formation of a diferulic acid sucrose ester (E), which differs clearly in the chromatographic properties from DFS (see Fig. 5). The splitting of the sucrose of this compound does not produce two monoferulic esters (compare the structure with TFS, Fig. 3). Thus, for substance E, the structure shown in Fig. 3 has to be proposed. Sucrose esters with only sinapic acid acyl moieties remain absolutely unaffected when incubated with either EA I or EA II. The sucrose esters which were formed enzymatically (see above; E, A, Fig. 3) do

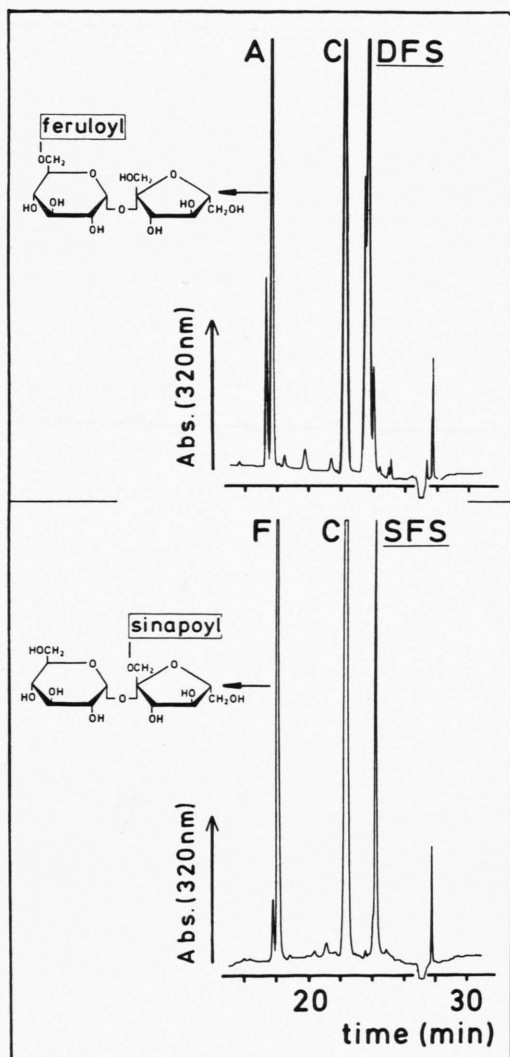


Fig. 4. HPLC-analyses of an enzyme assay with DFS and SFS as substrates and partially purified EA I (Chromatofocusing).

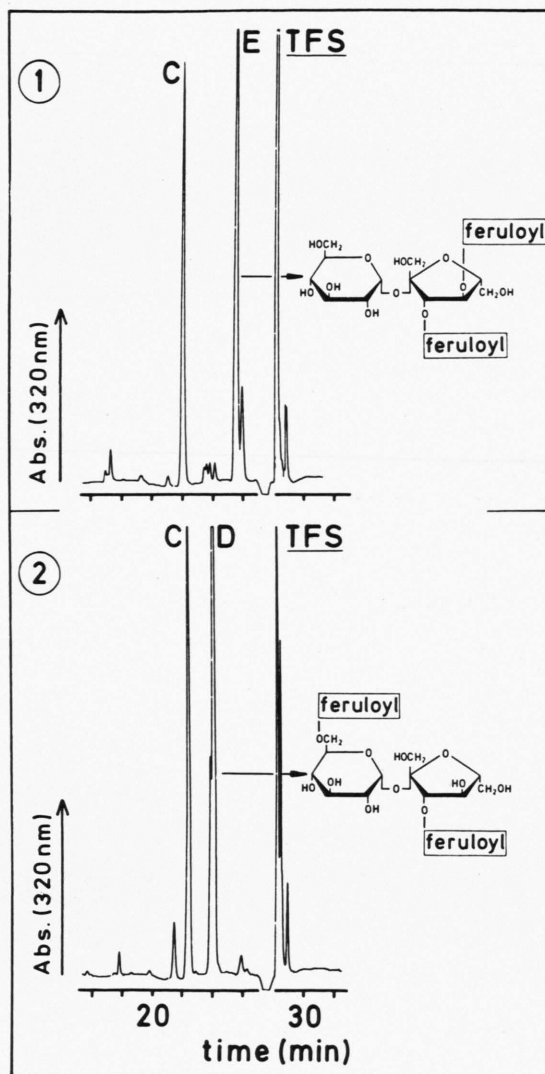


Fig. 5. HPLC-analyses of an enzyme assay in which TFS was hydrolyzed by partially purified EA I (①) and EA II (②) achieved by chromatofocusing.

not function as substrates for the two enzyme activities, although they contain ferulic acid as conjugation partner. In general, the results show that the specificity of EA I and EA II is related to the substitution pattern in the aromatic ring system, to the kind of the conjugation partners and to the sites of esterification.

#### *Some properties of EA I and EA II*

Product formation is linear up to 30 µg protein (partially purified EA I and EA II obtained after chromatofocusing) and up to 20 nmol of the substrates DFS and TFS, respectively. The IEPs with a pH 6.4 for EA I and 5.4 for EA II are remarkably different. Both enzymes show identical temperature optima of about 40 °C, but differ in their pH optima: EA I, pH 6.5 and EA II, pH 7.5. Addition of SH-reagents (β-mercaptoethanol, DTE, glutathione) does not influence the enzyme activities to a considerable extent.

#### **Discussion**

During recent years, an increasing number of esterases with a high specificity for genuine plant esters has been isolated and characterized [6–9]. As outlined in this report, anthers of *Tulipa* cv. Apeldoorn contain various esterases which are involved in the hydrolysis of DFS and TFS, both are accumulated in anthers. Following their pronounced substrate specificity it is to be postulated that these enzymes are closely related to the ferulic acid metabolism in the anthers. TFS is hydrolyzed into two diesters by four esterase activities obtained after protein fractionation by chromatofocusing.

The two esterase activities EA I and EA II exhibit a high substrate specificity. Only hydroxycinnamic acid sucrose esters with a ferulic acid moiety were accepted as substrates. While various sucrose esters (TFS, DFS, and SFS) could be hydrolyzed by EA I, the TFS was the poor substrate for EA II. Esterases from plant material with a high substrate specificity are described for the sinapine metabolism in *Raphanus sativus* [7]. The sinapine esterase exhibits a high specificity towards sinapoylcholine; cholinesters of other hydroxycinnamic acids were accepted as well.

Endogenous isoflavone malonylglucoside in chickpea, FGM and BGM were degraded by a specific

malonyl-esterase [9]. The partially purified esterase acts on both substrates at equal rates. Other ester substrates remain nearly unaffected. Pfützner and Stöckigt [8] reported the occurrence of a highly specific enzyme involved in biosynthesis of sarpagine/ajmaline type alkaloids in *Rauwolfia serpentina*. Among 15 structurally different esters, only polynuridine aldehyde (100%) and akuammidine aldehyde (49%) functioned as substrates for the esterase.

Another highly specific degradation of a natural constituent is described by Pensl and Sütfield [6] from *Tagetes patulae*. The thiophene derivative 3,4-diacetoxybutinylbithiophene is converted to 3,4-dihydroxybutinylbithiophene by subsequently reacting dithiophene acetate esterases.

In all these examples the esterases showed an expressed substrate affinity to natural substrates.

The hydrolysis of TFS and DFS has to be seen with regard to the ferulic acid metabolism. As a working hypothesis we assume that the *in vivo* accumulation of DFS is due to the EA II activity and DFS might be specifically metabolized by EA I. It should be taken into consideration that the monoferuloylsucrose formed enzymatically in *in vitro* experiments [A, Fig. 3] is possibly identical to the feruloylsucrose(mono) accumulated in anthers in an early developmental stage [11]. In this context it will be important to analyze the correlation between the *in vivo* accumulation of the three ferulic acid sucrose esters and the appearance of the esterase activities hydrolyzing DFS and TFS during anther development. Recent results (unpublished) indicate that these esterases show their highest activity when DFS and TFS reach their accumulation maximum. This correlation, and the fact that DFS and TFS are accumulated exclusively in the contents of the anther (= loculus material [11]), require further investigations on the localization of the esterases in the different parts of the anthers. The knowledge of a possibly tissue-specific localization of the substrates and the esterases might lead to a better understanding of the ferulic acid metabolism in the anthers in general.

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