

# Purification of a Trypsin Inhibitor Secreted by Embryogenic Carrot Cells

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## ABSTRACT

A protease inhibitor with a molecular weight of about 12,800 was purified to electrophoretic homogeneity from *Daucus carota* cells. The protease inhibitor was heat stable and inhibited trypsin but had no activity toward chymotrypsin or subtilisin. Nonembryogenic as well as embryogenic strains contained the inhibitor in similar amounts, but in the embryogenic strains the trypsin inhibitor was released from the cells and as a result accumulated in high concentrations in the culture medium, whereas no release of the trypsin inhibitor was found during cultivation of the nonembryogenic strains. Very low amounts of acid phosphatase or  $\alpha$ -mannosidase activity were found in the culture filtrate of both embryogenic and nonembryogenic strains, which suggest that the release of the inhibitor from embryogenic strains was not due to cell lysis.

Carrot cell cultures provide a simple system for studies on somatic embryogenesis in plants, since the differentiation process in these cultures can be easily manipulated by exogenously supplied auxin. In the presence of 2,4-D unorganized growth of the cells is promoted, while in its absence the carrot cells usually differentiate into somatic embryos (15, 20). Although embryo formation has been subjected to intense morphological as well as biochemical studies (6, 7, 10, 12, 20, 23) the regulation of this process is far from elucidated. This lack of understanding may in part be explained by the difficulties to obtain high yields of synchronously developing embryos. Many studies have focused on biochemical changes occurring during the switch from callus growth to embryo formation, such as altered rate of DNA, RNA, and protein synthesis (8) or quantitative changes in different enzyme activities (12, 23, 24). Furthermore, some unique properties of differentiating carrot cells have recently been described. Sung and Okimoto (21) reported that two polypeptides are synthesized exclusively during embryo formation and these polypeptides may therefore be used as markers for the differentiation process.

During embryo formation some carrot strains have the ability to inactivate the fungal toxins cycloheximide (22) and  $\alpha$ -amanitin (14) supplied to the culture medium. The mechanism for this inactivation has not been clarified, although the enzyme tyrosinase (phenoloxidase) has been proposed as responsible for degradation of  $\alpha$ -amanitin since high activity of this enzyme was observed exclusively during embryogenesis in these carrot strains (14).

It is well established that during prolonged culture in 2,4-D-containing medium, carrot cell strains generally lose their embryogenic capacity and become nonembryogenic (19). We have

used embryogenic and nonembryogenic carrot strains in order to characterize strains with the capacity to form embryos. In a previous paper (3) we have shown that activity of the enzyme phenoloxidase in carrot is restricted to embryogenic strains. Moreover, the activity of this enzyme decreases concomitant with a decline in embryogenic capacity of the strains, indicating that the presence of phenoloxidase activity is related to embryogenic potential (3). A nonembryogenic strain was also found to express protease activity far exceeding that of two embryogenic strains (2) and although protease inhibitors were present in the cells, the level of inhibitor activity did not differ between the tested strains. In this paper we report that nonembryogenic as well as embryogenic carrot strains contain a trypsin inhibitor which is secreted to the external milieu exclusively by the embryogenic strains. The trypsin inhibitor has also been purified and partially characterized.

## MATERIAL AND METHODS

**Cell Material.** Two embryogenic and two nonembryogenic cell strains of *Daucus carota* were maintained on the B<sub>5</sub>-medium of Gamborg *et al.* (9) containing 0.1 mg/L 2,4-D. Suspension cultures were initiated by dispersing about 2 g (fresh weight) of callus into 15 ml of liquid culture medium in 100 ml Erlenmeyer flasks, and these cultures were then subcultured every 4th d. Three d after subculturing, embryo formation was induced by washing the cells four times in 2,4-D-free culture medium. The washed cells were then incubated in this medium at a density of about 1 g (fresh weight) in 15 ml medium. All cultures were kept in darkness at 25°C.

**Viability Determination.** A stock solution of FDA<sup>2</sup> (5 mg/ml in acetone) was stored at -20°C. This solution was added to the cell suspension to give a final concentration of FDA of 0.01%. After 10 min at room temperature the cells were examined for fluorescence using a Zeiss Standard Universal microscope equipped with a HBO 200 W/4 mercury lamp, filter BG 12/38 as exciter filter, and filter 53 as the barrier filter.

**Preparation of Carrot Cell Extract.** Three d after subculturing in medium with or without 2,4-D, the cells were collected by centrifugation (1000g, 20 min, 4°C) and the culture filtrate was stored on ice. The cell pellet was washed once in 0.1 M Tris-HCl (pH 8.0) containing 1 mM PTU, and then homogenized in 10 ml of this buffer with a glass piston homogenizer. The homogenate was centrifuged (10,000g, 20 min, 4°C) and the resulting supernatant was used as enzyme source or after incubation for

<sup>2</sup> Abbreviations: FDA, fluorescein diacetate; Bz-Ile-Glu-( $\gamma$ -OR)-Gly-Gly-Arg-pNA-HCl, *N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-*p*-nitroanilide hydrochloride methyl ester; CNBr, cyanobromide; HAc, acetic acid; Meo-Suc-Arg-Pro-Tyr-pNA-HCl, 3-carbomethoxypropionyl-L-arginyl-L-propyl-L-tyrosine-*p*-nitroanilide hydrochloride; pNA, *p*-nitroanilide; pNP, *p*-nitrophenol; PTU, phenylthiourea; STI, soybean trypsin inhibitor.

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15 min at 60°C and cooling on ice, as source of protease inhibitor.

**Assay of Protease Inhibitor Activity.** The inhibitor activity was assayed by incubating 50  $\mu$ l enzyme (dissolved in 0.1 M Tris-HCl, pH 8.0) and 50  $\mu$ l inhibitor preparation (purified inhibitor, crude cell extract, or culture filtrate) for 20 min at 22°C prior to the addition of 400  $\mu$ l 0.1 M Tris-HCl buffer (pH 8.0) and 100  $\mu$ l 2 mM synthetic peptide (Kabi Vitrum, Stockholm, Sweden) as substrate. The chromogenic peptides used were: Bz-Ile-Glu-( $\gamma$ -OR)-Glu-Gly-Arg-pNA·HCl (S-2222) for trypsin (Sigma EC 3.4.21.4) and Meo-Suc-Arg-Pro-Tyr-pNA·HCl (S-2586) for chymotrypsin (EC 3.4.21.1) and subtilisin (Sigma EC 3.4.21.14) and the peptides were prepared as previously described (2). For controls, 50  $\mu$ l buffer or fresh culture medium were used instead of the cell extracts or culture filtrates. The reaction mixture was incubated at 30°C and the enzyme reaction was terminated after 30 min for trypsin assays, and 30 to 60 min for chymotrypsin and subtilisin assays by addition of 100  $\mu$ l 50% HAc. After centrifugation (3000g, 10 min) the released pNA was determined in the resulting supernatant at 405 nm. The reaction rates were linear during the incubation time and proportional to the enzyme concentration. The amount of inhibitor is expressed as ng active trypsin inhibited per mg protein of the purified inhibitor, cell extract, or culture filtrate. The trypsin inhibitor activity was estimated at 50% inhibition of the trypsin activity in the reaction mixture and the fraction of active trypsin in the preparation used was estimated by active site titration according to Chase and Shaw (4).

**Assay of Acid Phosphatase Activity.** Acid phosphatase activity was measured by incubating 25  $\mu$ l cell extract or culture filtrate, 125  $\mu$ l 0.09 M Na-citrate buffer (pH 5.1) and 125  $\mu$ l Sigma 104 phosphatase substrate (4 mg/ml) at 37°C for 30 min. The reaction was terminated by addition of 1.25 ml 0.02 M NaOH and the released pNP was determined at 410 nm. Enzyme activity is expressed as nmol pNP released per min and mg protein.

**Assay of  $\alpha$ -Mannosidase Activity.**  $\alpha$ -Mannosidase activity was assayed by incubating 25  $\mu$ l cell extract or culture filtrate with 250  $\mu$ l 0.66 mM *p*-nitrophenyl- $\alpha$ -D-mannopyranoside (Sigma) in 90 mM sodium citrate buffer (pH 4.5) for 60 min at 35°C. The reaction was terminated by addition of 1.25 ml 0.2 M NaOH and released pNP was determined at 410 nm. Enzyme activity is expressed as nmol pNP released per min and mg protein.

**Protein Determination.** Protein was determined according to Bradford (1) using BSA (Sigma) as standard.

**Purification of Trypsin Inhibitor.** The trypsin inhibitor was purified from the culture filtrate of the embryogenic carrot cells. After 5 d of culturing, the cells were removed by centrifugation (1000g, 5 min) and the culture filtrate was centrifuged a second time at 10,000g (20 min, 4°C), and to the resulting supernatant

(about 130 ml)  $(\text{NH}_4)_2\text{SO}_4$  was added to 80% saturation. After about 2 h at 4°C the precipitate was collected by centrifugation (10,000g, 20 min, 4°C) and dissolved in 5 ml 0.01 M Tris-HCl (pH 8.5) (buffer 1) and extensively dialyzed against this buffer. The precipitate formed during dialysis was removed by centrifugation (50,000g, 20 min, 4°C). The resulting supernatant was placed in a boiling water bath for 2 min and then centrifuged at 3000g for 5 min before applying to a DEAE-cellulose column (1.6  $\times$  20 cm) equilibrated with buffer 1. The column was washed with the equilibrating buffer and the trypsin inhibitor activity was eluted as a single peak with 0.15 M NaCl in buffer 1 (flow rate 20 ml/h, 1.9 ml/fraction). The fractions containing trypsin inhibitor activity were pooled (about 15 ml) and applied to a trypsin-Sepharose column (1.6  $\times$  1.5 cm) equilibrated with 0.1 M Tris-HCl (pH 8.5) containing 0.5 M NaCl (buffer 2). The trypsin-Sepharose column was prepared by coupling about 4.5 mg of active trypsin (Sigma) to 1 g CNBr-activated Sepharose (Pharmacia) according to the manufacturer's instructions. After extensive washing with buffer 2, the trypsin inhibitor was eluted with 0.5 M NaCl (pH 2.0) (flow rate 10 ml/h, 1.2 ml/fraction). The pH of the eluted fractions was adjusted to 8.0. The fractions containing trypsin inhibitor activity from the trypsin-Sepharose column were pooled (13 ml), extensively dialyzed against distilled water, and lyophilized. The lyophilized trypsin inhibitor was dissolved in 1 ml of 0.05 M Tris-HCl (pH 8.5) containing 0.02 M NaCl (buffer 3) and applied to a Sephadex G-50 (Pharmacia) gel filtration column (1.5  $\times$  90 cm) equilibrated with buffer 3. A single peak of trypsin inhibitor activity was eluted with the equilibrating buffer (flow rate 9.0 ml/h, 2.25 ml/fraction) and the inhibitor containing fractions were pooled, dialyzed against distilled H<sub>2</sub>O, and lyophilized. For mol wt determination, ribonuclease A (13,700), soybean trypsin inhibitor (21,000), and chymotrypsinogen (25,000) were used as standard proteins for calibration of the Sephadex column. The void volume was determined using Blue Dextran 2000 (Pharmacia). For determination of the mol wt as well as the purity of the trypsin inhibitor, SDS gel electrophoresis was carried out according to Laemmli (11) at pH 8.3 using a 15% polyacrylamide gel containing 0.1% SDS under nonreduced or reduced conditions. The lyophilized preparation after Sephadex G-50 was dissolved in distilled water and 50  $\mu$ l of this preparation containing 10  $\mu$ g protein was applied to the gel. The reference proteins for mol wt determination were phosphorylase *b* (94,000), albumin (67,000), ovalbumin (43,000), trypsin inhibitor (20,100), and  $\alpha$ -lactalbumin (14,400) (Pharmacia low mol wt calibration kit). After electrophoresis (50 mamp, 4 h) the gel was fixed in 10% HAc dissolved in 50% methanol overnight and then stained in 0.05% Coomassie brilliant blue R250.

Table 1. Intracellular and Extracellular Activity of Trypsin Inhibitor in Nonembryogenic or Embryogenic Strains of *D. carota* after 3 d Culturing with or without 2,4-D in Culture Medium

Mean value  $\pm$ SD of 4 experiments.

Cell Strain	Culture Medium	Specific Inhibitor Activity		
		Intracellular	Extracellular	Total
<i>ng trypsin inhibited mg<sup>-1</sup> protein</i>				
<i>Nonembryogenic</i>				
NE-1	+2,4-D	22.3 $\pm$ 4.3	0	22.0 $\pm$ 4.2
	-2,4-D	18.4 $\pm$ 8.5	0	18.2 $\pm$ 8.4
NE-2	+2,4-D	31.2 $\pm$ 6.7	0	31.1 $\pm$ 6.5
	-2,4-D	27.6 $\pm$ 9.4	0	27.6 $\pm$ 9.2
<i>Embryogenic</i>				
E-1	+2,4-D	12.1 $\pm$ 5.2	1321 $\pm$ 205	32.6 $\pm$ 3.6
	-2,4-D	8.4 $\pm$ 1.4	1304 $\pm$ 212	33.3 $\pm$ 4.4
E-2	+2,4-D	12.8 $\pm$ 2.5	1240 $\pm$ 86	23.4 $\pm$ 2.4
	-2,4-D	14.3 $\pm$ 3.0	2232 $\pm$ 544	33.4 $\pm$ 6.7

Table II. Intracellular and Extracellular Activity of Trypsin Inhibitor, Acid Phosphatase, and  $\alpha$ -Mannosidase in Nonembryogenic or Embryogenic Cell Strain of *D. carota* after 3 d Culturing with or without 2,4-D in Culture Medium

The activity represents total activity in 5 ml cell suspension. IC = intracellular, EC = extracellular. Mean value  $\pm$ SD of 5 experiments.

Cell Strain	Culture Medium	Protein		Trypsin Inhibitor Activity		Acid Phosphatase Activity		$\alpha$ -Mannosidase Activity	
		IC	EC	IC	EC	IC	EC	IC	EC
		mg		ng trypsin inhibited		nmol pNP min <sup>-1</sup>		nmol pNP min <sup>-1</sup>	
Nonembryogenic NE-1	+2,4-D	4.58 $\pm$ 0.30	0.05 $\pm$ 0.01	94 $\pm$ 20	0	3375 $\pm$ 880	981 $\pm$ 218	119 $\pm$ 5	14 $\pm$ 2
	-2,4-D	5.81 $\pm$ 0.71	0.07 $\pm$ 0.02	92 $\pm$ 24	0	3516 $\pm$ 648	863 $\pm$ 180	139 $\pm$ 8	8 $\pm$ 1
Embryogenic E-2	+2,4-D	3.36 $\pm$ 0.31	0.03 $\pm$ 0.02	46 $\pm$ 4	45 $\pm$ 7	1236 $\pm$ 63	278 $\pm$ 50	136 $\pm$ 5	7 $\pm$ 2
	-2,4-D	3.97 $\pm$ 0.61	0.03 $\pm$ 0.02	58 $\pm$ 6	74 $\pm$ 15	1508 $\pm$ 293	208 $\pm$ 40	133 $\pm$ 5	9 $\pm$ 3

Table III. Purification of Trypsin Inhibitor from Culture Filtrate of Embryogenic Carrot Cells

Fraction	Volume	Total Activity	Protein	Specific Activity	Yield	Purification
	ml	ng trypsin inhibited	mg	ng trypsin inhibited mg <sup>-1</sup> protein	%	-fold
Culture filtrate	130	12168	6.5	1872	100	1
Ammonium sulfate fraction	4.7	7002	1.7	4119	58	2.2
DEAE-cellulose	16.5	6178	0.49	12607	51	6.7
Trypsin-Sepharose	12.6	2654	0.008	331695	22	177
Sephadex G-50	3.9	1521	0.002	760500	12	406

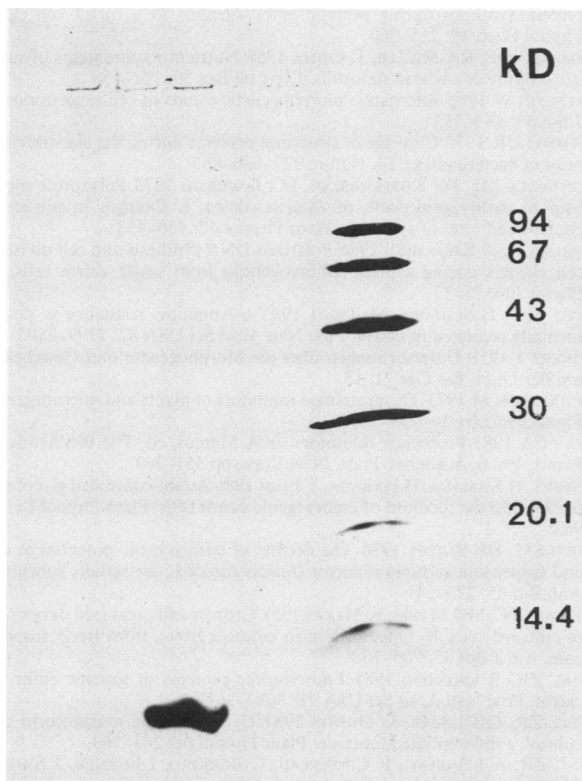


FIG. 1. SDS-PAGE under reducing conditions of the purified trypsin inhibitor preparation. The mol wt of protein standards are indicated on the right.

RESULTS

Trypsin inhibitor activity was detected in both the nonembryogenic and embryogenic strains of *D. carota* and the specific

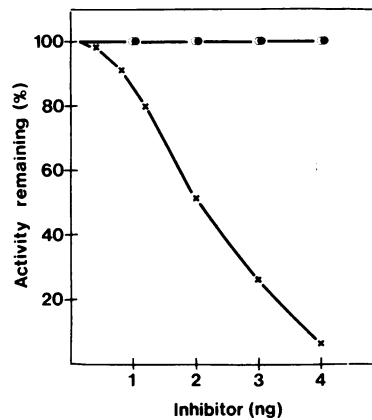


FIG. 2. Specificity of the purified trypsin inhibitor from *D. carota*. The specificity was assayed by incubating 50  $\mu$ l of the enzyme with different amounts of the purified inhibitor preparation for 20 min at 22°C. The 100  $\mu$ l of substrate and 0.1 M Tris-HCl (pH 8.0) were added to a final volume of 600  $\mu$ l. The reaction was terminated by addition of 100  $\mu$ l 50% HAc. The amount of enzyme in the reaction mixture was (x) trypsin, 3.9 ng; (●) subtilisin, 2.5 ng; (○) chymotrypsin 5 ng.

inhibitor activity of the cells did not differ significantly between strains with different capacity for differentiation (Table I). The inhibitor activity did not change during the differentiation process, since isolated embryos showed the same inhibitor activity as undifferentiated cell aggregates (data not shown). However, a considerable amount of the trypsin inhibitor activity was found in the culture filtrate of the embryogenic cell cultures, whereas no inhibitor activity was released from the nonembryogenic strains into the surrounding medium (Table I). To test if the trypsin inhibitor activity of the culture filtrate was due to cell lysis, we assayed the extracellular and intracellular activities of two reference enzymes, acid phosphatase and  $\alpha$ -mannosidase. About 10 to 20% of the total acid phosphatase activity was found outside the cells in the embryogenic as well as in the nonembry-

ogenic cultures (Table II), and only 0 to 10% of the  $\alpha$ -mannosidase activity was extracellular. On the other hand, 50 to 60% of the trypsin inhibitor was released from the embryogenic strains whereas no release could be noted from the nonembryogenic strains (Table II). The trypsin inhibitor, acid phosphatase and  $\alpha$ -mannosidase activities were all stable in the culture filtrate for at least 5 d when incubated at 25°C, showing that no degradation of these extracellular activities occurred during the incubation time. Moreover, staining of the cells with FDA showed that 95 to 100% of the cells had an intact plasma membrane, which was also indicated by the low amount of protein detected outside the cells. These data taken together suggest that the release of trypsin inhibitor activity from the embryogenic strains is a specific secretion process and not due to cell lysis.

For further characterization, the trypsin inhibitor was purified to electrophoretic homogeneity starting from the culture filtrate of one embryogenic carrot strain and a typical purification of the trypsin inhibitor is summarized in Table III. SDS-PAGE under reducing or nonreducing conditions showed a single polypeptide with a mol wt of 12,800 (Fig. 1). The mol wt of an unreduced inhibitor sample as estimated by gel sieving on a Sephadex G-50 column was 16,000 (the  $K_{av}$ -values were; ribonuclease A 0.268, carrot trypsin inhibitor 0.200, soybean trypsin inhibitor 0.084, and chymotrypsinogen 0.051). The purified as well as the crude inhibitor resisted incubation for 5 min in a boiling water bath, whereas after 15 min 40 to 50% of the activity in both preparations was lost. After exposure to pH values between 2 and 12 for 5 h at 22°C the inhibitor activity remained constant. The inhibitor strongly inhibited trypsin ( $K_i$ ,  $1.5 \cdot 10^{-9}$ M), whereas no detectable inhibition of chymotrypsin or subtilisin could be observed (Fig. 2).

## DISCUSSION

Several protease inhibitors have been purified and extensively studied from a variety of plant species (16, 17) and in carrot tissues trypsin inhibitor activity has been described (5), but as yet no purification or characterization has been made. In this paper, we describe the purification to electrophoretic homogeneity of a trypsin inhibitor from carrot cells consisting of one single polypeptide chain with a mol wt of about 12,800 as judged by SDS-PAGE. We have in a previous paper shown that the trypsin-like protease activity in a nonembryogenic carrot cell strain far exceeded that of embryogenic strains (2). All strains were also found to contain trypsin inhibitor activity, but no considerable difference in the amount of trypsin inhibitor activity could be detected between the strains and, therefore, could not account for the higher protease activity of the nonembryogenic strain. Although the total amount of trypsin inhibitor activity was similar in the different cell strains, extracellular activity was confined to the embryogenic strains. Our results indicate that the release of trypsin inhibitor is due to a specific secretion process in these strains, since the activity of the marker enzymes acid phosphatase and  $\alpha$ -mannosidase was very low in the culture filtrate of both nonembryogenic and embryogenic strains. Furthermore, the low amount of extracellular protein and the high viability of the cells shown by FDA-staining suggest that no extensive cell lysis occurred. However, we have not yet been able to show the mechanism by which the trypsin inhibitor is released into the surrounding medium. Recently, Satoh *et al.* (18) showed that a glycoprotein with the mol wt of 57,000 was released from embryogenic carrot cells only in the presence of 2,4-D. In our study, though, the trypsin inhibitor was secreted from the cells during embryo formation as well as during callus growth, show-

ing that the auxin was not the responsible agent for the secretion.

It is generally believed that 2,4-D does not prevent the induction of embryo formation, but rather inhibits the normal development of the embryos. Polarized divisions in single cells and the development of embryogenic cell clusters have been shown in the presence of 2,4-D (13) as well as the formation of globular stage embryos (21). Therefore, in embryogenic cell strains differentiation has already been initiated, but very few cell clusters will mature into embryos in the presence of 2,4-D. Presently we can not propose any biological function for the secretion, or the presence of the trypsin inhibitor in the external milieu of embryogenic carrot cell strains, but merely indicate that this is an inherent property of cells with the capacity to form embryos.

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