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Purification and Properties of Intracellular Exo- and Endoinlulinases from *Aspergillus niger* Strain 12

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Abstract

Exo- and endoinulinases were extracted from mycelia of *Aspergillus niger* strain 12 using quartz sand and purified by DEAE-Cellulofine A-500, Sephadex G-100, and Sephadex G-200 chromatographies. The intracellular enzymes were homogeneous as judged by SDS-polyacrylamide gel electrophoresis. The purified exoinulinase P-II had specific activities of 6.6 U/mg toward inulin and 22 U/mg toward sucrose. The endoinulinase P-III showed 108 U/mg toward inulin, but no activity toward sucrose. M_rs of exoinulinase P-II and endoinulinase P-III were determined by gel filtration using Sephadex G-200 as 47 and 56 kDa, respectively. Optimal pH and temperature for enzyme activity were pH 5.0 and 55 ℃ for P-II, and pH 5.3 and 45 ℃ for P-III. Both the enzymes were activated by Mn^{2+}, and inactivated by Ag^{+}, Hg^{2+}, or *p*-chloromercuribenzoate. Inulinases P-II and P-III exhibited apparent K_m values of 5.8 and 0.80 mM, respectively.

Key words: *Aspergillus niger*, Intracellular enzyme, Inulin, Inulinase

Introduction

A polyfructan, inulin, is present as a reserve carbohydrate in the roots and tubers of plants like Jerusalem artichoke, chicory, and dahlia[1]. It consists of linear chains of β-2, 1-linked D-fructofuranose molecules terminated by a glucose residue attached through a sucrose-type linkage at the reducing end.

Inulinases occur in bacteria, yeasts, filamentous fungi, and plants. Most microbial inulinases are inducible and exo-acting enzymes. In our laboratory, a filamentous fungus *Aspergillus niger* strain 12 has been shown to produce three extracellular inulinases constitutively: two exoinulinases (P-I and P-II) and a single endoinulinase (P-III)[2-5]. Exoinulinases (β-D-fructan fructohydrolase, EC 3.2.1.80) split off terminal fructose units successively from the nonreducing end of the inulin

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Abbreviations: DP, degree of polymerization; F, fructose; F_2, inulobiose; F_3, inulotriose; F_4, inulotetraose; G, glucose; GF, sucrose; GF_2, 1-kestose; GF_3, nystose; GF_4, 1^F-β-fructofuranosylnystose; IEF, isoelectric focusing; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

molecule, but they also hydrolyze the 2,1-β-D-fructofuranosidic linkages in sucrose and raffinose. On the other hand, endoinulinases (2,1-β-D-fructan fructanohydrolase, EC 3.2.1.7) are specific for inulin and hydrolyze the internal linkages in inulin to yield inulotriose, -tetraose, and -pentaose as the main products. Recently, we generated an endoinulinase-hyperproducing mutant *A. niger* 817 from the wild-type strain *A. niger* 12[6]. Extracellular inulinases from the *A. niger* 817 were successfully applied to production of fuel ethanol[7], fructose syrup[8], and inulo-oligosaccharides[9] from inulin.

It would be desirable to know the properties of intracellular inulinases for studying the secretion in a fungal system. However, intracellular inulinases have been described only for the exo-acting enzymes from yeasts such as *Saccharomyces fragilis*[10], *Kluyveromyces fragilis*[11], and *Candida kefyr*[12], and a filamentous fungus *Penicillium* sp. strain 1[13]. In this article, intracellular exo- and endoinulinases from *A. niger* 12 were purified and their enzymatic properties were compared with those of the extracellular equivalents.

Materials and Methods

Organism and culture conditions.

A. niger 12 from our laboratory collection was maintained on agar slants (pH 5.5), which contained (per liter) 10 g of inulin, 5 g of peptone, 3 g of $(NH_4)_2HPO_4$, 0.5 g of KCl, 0.5 g of $MgSO_4 \cdot 7H_2O$, 10 mg of $FeSO_4 \cdot 7H_2O$, and 20 g of agar[2]. For inulinase production, it was grown in submerged culture at 30 ℃ for 5 days, using a liquid medium (pH 4.5), which contained 30 g of fructose, 20 g of corn steep liquor, 12 g of $NH_4H_2PO_4$, 0.7 g of KCl, 0.5 g of $MgSO_4 \cdot 7H_2O$, and 10 mg of $FeSO_4 \cdot 7H_2O$, as previously described[2].

Enzyme and protein assays.

Exoinulinases were distinguishable from endoinulinases by their ability to hydrolyze sucrose[3,4]. For this reason, inulinase activity [I] is commonly compared with the invertase activity [S] displayed by the same enzyme preparation[1]. Inulinase and invertase activities were assayed by measuring reducing sugars released from inulin and sucrose, respectively, after incubation at 40 ℃ for 30 min, as described previously[6]. Calibration curves were prepared with D-fructose for assay of inulinase activity and an equimolar mixture of D-fructose and D-glucose for assay of invertase activity. One unit of inulinase activity was defined as the amount of enzyme that liberated 1.0 μmol of fructose equivalent from inulin per min. One unit of invertase activity was defined as the amount of enzyme that hydrolyzed 1.0 μmol of sucrose per min. Protein content was determined either by the method of Lowry *et al.*[14] or by A_{280}, using bovine serum albumin as the standard.

Extraction and purification of intracellular inulinases.

Unless otherwise indicated, all purification steps were performed at 4 ℃. The submerged culture was filtered through three layers of cheesecloth. The mycelia were washed with deionized water until no inulinase activity was detected in the washings. The washed mycelia were suspended in 300 ml of 50 mM acetate buffer (pH 5.0) and ground in the presence of quartz sand with a mortar and pestle. Additional 300 ml of the same buffer was added to the homogenate. The mixture was centrifuged at 6,000 × *g* for 10 min to pellet cell debris and the cell-free extract was collected. This extraction procedure was repeated twice. The pooled extract was concentrated with polyethylene glycol (average MW, 6000), and proteins were precipitated by adding solid ammonium sulfate to give 70 % saturation. The precipitate was recovered by centrifugation at 15,000 × *g* for 45 min, dissolved in a small volume of 0.1 M acetate buffer (pH 5.0), and dialyzed overnight against 20 mM acetate buffer (pH 5.0). Ice-cold

ethanol was added to the enzyme solution for fractionation as described previously[15]. The precipitates obtained by 0 to 60 % (v/v) and 60 to 75% (v/v) ethanol were individually dissolved in 20 ml of 20 mM acetate buffer (pH 6.0) and dialyzed for a day against the same buffer. The enzyme solutions from 0 to 60 % (v/v) and 60 to 75 % (v/v) fractions were subjected to chromatographies of DEAE-Cellulofine A-500 (Seikagaku Kogyo, Tokyo), Sephadex G-100, and Sephadex G-200 (Pharmacia Biotech, Uppsala, Sweden) for purification of the intracellular exo- and endoinulinases, respectively.

Analytical methods for proteins.

Endoinulinase P-III (about 1 mg) was hydrolyzed *in vacuo* with 6 N HCl at 110 ℃ for 24 h and the amino acid composition of the residues was determined by the procedure of Spackman *et al.*[16] with a Hitachi amino acid analyzer (model 835).

SDS-PAGE was carried out in 8 % (w/v) polyacrylamide gel by using 25 mM Tris-glycine buffer (pH 8.3) containing 0.1 % (w/v) SDS[17]. Gels were stained for protein with 0.1 % (w/v) Coomassie brilliant blue R-250. Apparent M_rs were determined by gel filtration using Sephadex G-200.

Preparative IEF was performed in a 4 % (w/v) granulated gel (Ultrodex; LKB, Uppsala, Sweden) according to the manual of LKB 2117 Multiphor for 16 h at 8 W constant power and 4 ℃ using 5 % (w/v) ampholyte (pH range 4 to 6.5). After focusing, the gel was cut into 37 slices with a fractionating grid. The pH gradient was determined by measuring the pH of each slice. Extracts from each gel slice with 5 ml of 0.1 M acetate buffer (pH 6.0) were used for determination of enzyme activities and protein contents.

Determination of kinetic parameters.

The initial velocities of inulin hydrolysis by 27 and 1.55 g/ml of intracellular enzymes P-II and P-III were determined at various substrate concentrations ranging from 0.125 to 5.0 mM in 20 mM acetate buffer (pH 5.0) at 40 ℃. The average molecular weight of inulin was assumed to be 5400 from the average DP. Michaelis-Menten constants (K_m) were estimated using a Lineweaver-Burk plot.

Analysis of hydrolysis products of inulin.

Total sugars of inulin were determined as reducing sugars after acid hydrolysis in 0.1 N HCl. The extents of inulin hydrolysis (%) were calculated as (μmol of reducing sugars released/μmol of fructose equivalents of inulin) × 100. D-Glucose was assayed colorimetrically with glucose oxidase and peroxidase (Boehringer Mannheim GmbH, Mannheim Germany)[8]. D-Fructose was determined as the difference between the amounts of total reducing sugars and D-glucose. TLC was performed on HPTLC pre-coated silica gel 60 plates (Merck AG, Darmstadt, Germany)[6]. Paper chromatography was carried out on Toyo No. 50 filter paper (40 × 40 cm)[5].

Chemicals.

Inulin derived from dahlia tubers, bacterial levan from *Erwinia herbicola*, and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fructose, glucose, sucrose, raffinose, melezitose, and quartz sand were purchased from Wako Pure Chemical Industries (Osaka). Fructo- and inulo-oligosaccharides (GF_2, GF_3, GF_4, F_2, F_3, and F_4) were supplied by Meiji Seika Kaisha Ltd. (Tokyo). All other chemicals were of reagent grade.

Results

Purification of exo- and endoinulinases

The procedure for the purification for intracellular inulinases P-II and P-III is summarized in Tables 1 and 2. The P-II and P-III were purified 105- and 146-fold over the culture filtrate, with the yield of 0.04 and 2.3 %, respectively. The

Table 1. Summary of purification procedure for P-II

Step	Total protein (mg)	Total units with inulin	Total units with sucrose	Units/mg protein with inulin	Units/mg protein with sucrose	I/S	Yield (%)
Crude extracts	64,871	47,957	2,609	0.74	0.04	18.4	100
$(NH_4)_2SO_4$(0-70 % saturation)	2,491	32,535	1,913	13.06	0.77	17.0	68.0
Concentrate	2,056	30,984	1,830	15.07	0.89	16.9	64.7
Ethanol precipitate(0-60 %, v/v)	1,309	8,822	1,637	6.74	1.25	5.40	18.40
DEAE-Cellulose(Stepwise)	121.6	167.6	689.6	1.38	5.67	0.24	0.35
Sephadex G-100	104.4	116.8	274.5	1.12	2.63	0.43	0.24
DEAE-Cellulose(Gradient)	40.5	46.0	141.0	1.36	3.48	0.32	0.10
DEAE-Cellulose(Gradient)	23.4	43.4	135.4	1.85	5.79	0.32	0.09
Sephadex G-200	18.8	37.0	116.5	1.97	6.20	0.32	0.08
Sephadex G-100	6.9	28.9	94.8	4.19	13.65	0.31	0.06
Sephadex G-100	3.1	20.5	68.3	6.61	22.03	0.30	0.04

Table 2. Summary of purification procedure for P-III

Step	Total protein (mg)	Total units with inulin	Total units with sucrose	Units/mg protein with inulin	Units/mg protein with sucrose	I/S	Yield (%)
Crude extracts	64,871	47,957	2,609	0.74	0.04	18.4	100
$(NH_4)_2SO_4$(0-70 % saturation)	2,491	32,535	1,913	13.06	0.77	17.0	68.0
Concentrate	2,056	30,984	1,830	15.07	0.89	16.9	64.7
Ethanol precipitate(60-75 %, v/v)	245	16,200	8	66.12	0.03	1,944.8	33.9
DEAE-Cellulose(Stepwise)	121	15,678	0.3	129.14	0.003	47,508.6	32.8
Sephadex G-100	74	13,322	0	179.78	0	–	27.8
DEAE-Cellulose(Gradient)	63	8,889		142.22		–	18.6
Sephadex G-200	40	6,920		172.58		–	14.5
DEAE-Cellulose	10	1,120		108.21		–	2.3

enzymes were homogeneous as judged by SDS-PAGE. The purified exoinulinase P-II had specific activities of 6.6 U/mg toward inulin and 22 U/mg toward sucrose (I/S ratio, 0.30). The endoinulinase P-III showed 108 U / mg toward inulin, but no activity toward sucrose. The M_rs of inulinases P-II and P-III were determined by gel filtration using Sephadex G-200 as 47 and 56 kDa, respectively (Fig. 1). The M_r of the intracellular endoinulinase P-III was close to 54 kDa of the extracellular enzyme P-III, whereas the M_r of the intracellular exoinulinase P-II was smaller than those of the extracellular enzymes P-I (72 kDa) and P-II (59 kDa).

Further physicochemical properties were studied for the endoinulinase P-III. The ultraviolet

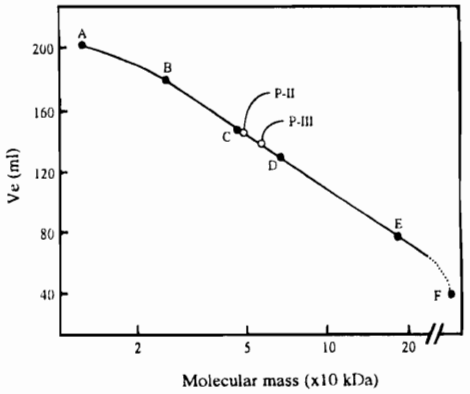

Fig. 1. Determination of molecular masses of inulinases P-II and P-III from *A. niger* 12 on a Sephadex G-200 gel-filtration column.

Standard molecular mass markers: A, cytochrome (12.4 kDa); B, chymotrypsinogen (25 kDa); C, ovalbumin (45 kDa); D, albumin (67 kDa); E, γ -globulin (160 kDa); F, Blue Dextran (2000 kDa). Elution volume (Ve) was plotted against decadic logarithm of molecular masses for proteins.

absorption spectrum of endoinulinase P-III in 20 mM acetate buffer (pH 6.0) presented a single absorption peak having a maximum at 280 nm and minimum at 250 nm (data not shown). Intracellular endoinulinase P-III was abundant in hydroxyamino acids such as Thr (10.7 %) and Ser (9.3 %). The amino acid composition of intracellular endoinulinase P-III was in fairly good agreement with the overall amino acid composition deduced from the *A. niger* endoinulinase gene[18]. The isoelectric point was pH 3.6, close to the pH 3.7 for the extracellular equivalent[19].

Enzymatic properties

Enzymatic properties of exo- and endoinulinases are summarized in Table 3. Effects of pH and temperature on activity and stability of intracellular inulinases were studied under the conditions described previously[6]. Inulinases P-II and P-III showed maximum activities at pH 5.0 and 5.3, respectively. Inulinase activities were stable for 24 h over pH ranges from 3.2 to 7.0 for P-II and from 5.0 to 7.0 for P-III. The intracellular enzymes showed maximum activities at 55 ℃ for P-I and 45 ℃ for P-II. Inulinases P-II and P-III were stable for 30 min up to 50 and 60 ℃, respectively, with no loss of activity. Complete inactivation was observed when P-II and P-III were treated at 70 and 80 ℃, respectively.

The effects of various potential enzyme inhibitors and metal ions on the activity of intracellular inulinases were examined at a final concentration of 1.0 mM, as previously described[6]. In common with extracellular inulinases from *A. niger* 12[3-5] and *Penicillium* sp. 1[20], Mn^{2+} appeared to stimulate the activity of the inulinases P-II (135 %) and P-III (132 %). Ag^{+} or Hg^{2+} caused complete inactivation of inulinase activity and an appreciable loss of activity was observed for *p*-chloromercuribenzoate (27 % for P-II and 20 % for P-III), EDTA (85 % for P-II and 42 % for P-III) or Fe^{3+} (35 % for P-II and 16 % for P-III).

The affinity of the purified enzymes for inulin was examined with a Lineweaver-Burk plot. Both enzymes showed Michaelis-Menten-type kinetics with inulin as the substrate at 40 ℃ and pH 5.0. Inulinases P-II and P-III exhibited apparent K_m values of 5.8 and 0.80 mM, respectively.

Substrate specificity

The substrate specificity of intracellular inulinases P-II and P-III was examined for fructofuranosides such as inulin, sucrose, raffinose, melezitose, and bacterial levan (a β-2,6 fructan) (Table 4). The reaction mixture, consisting of 0.5 ml of each substrate (0.5 %, w/v) and an equal volume of the suitably diluted enzyme solution, was incubated at 40 ℃ for 30 min, and the liberated reducing sugars were measured as described previously[6]. Inulinase P-II hydrolyzed inulin (100 %), sucrose (348 %), and raffinose (86 %), whereas levan were not

Table 3. Properties of inulinases P-II and P-III

Properties	P-II	P-III
Molecular mass(kDa)[a]	47	56
pH optimum (stability)	5.0 (3.2-7.0)	5.3 (5.0-7.0)
Temperature optimum (stability)	55℃ (<50℃)	45℃ (<60℃)
Inhibitor	Hg^{2+}, Ag^{+}, *p*CMB[b]	Hg^{2+}, Ag^{+}, *p*CMB[b]
Activator	Mn^{2+}	Mn^{2+}
K_m for inulin(mM)	5.8	0.8

[a] gel filtration by Sephadex G-200
[b] *p*-chloromercuribenzoate

Table 4. Substrate specificity

Substrate	Relative activity (%)	
	P-II	P-III
Inulin	100	100
Sucrose	348	0
Raffinose	86	0
Melezitose	0	0
Levan	0	0

hydrolyzed. In order to determine from which end the inulin molecule is hydrolyzed by the exo-enzyme, melezitose (3-*O*-α-D-glucopyranosyl-β-D-fructofuranosyl-α-D-glucopyranoside) was used as substrate. This trisaccharide has the same terminal configuration as inulin, but the centrally located fructose is protected from terminal hydrolysis by a second glucosyl residue, attached to the fructose at the 3-position. The exoinulinase had no hydrolytic activity toward melezitose, showing the absence of α-glucosidase activity. This indicated that inulin is hydrolyzed from the fructose end by a sequential hydrolysis of fructose molecules.

Inulinase P-III was active toward inulin, but did not release any detectable reducing sugars from sucrose, raffinose, or levan. Thus, the inulinase P-III was specific for internal 2,1-β-D-fructofuranosidic linkages in inulin.

Reaction products of inulin hydrolysis by intracellular inulinases

During the course of inulin hydrolysis by inulinases P-II and P-III, aliquots (2 μl) of the reaction mixture were periodically withdrawn and analyzed for the hydrolysis products by paper chromatography in order to characterize the mode of action of the enzymes (Fig. 2). When inulin was treated with inulinase P-II, fructose was the only reaction product detectable. Random attack by inulinase P-III on inulin yielded a series of oligosaccharides with a DP of 3 and above during the first 5 h of incubation. Inulotriose (F_3), -tetraose (F_4), -pentaose (F_5), and -hexaose (F_6) were liberated as the main

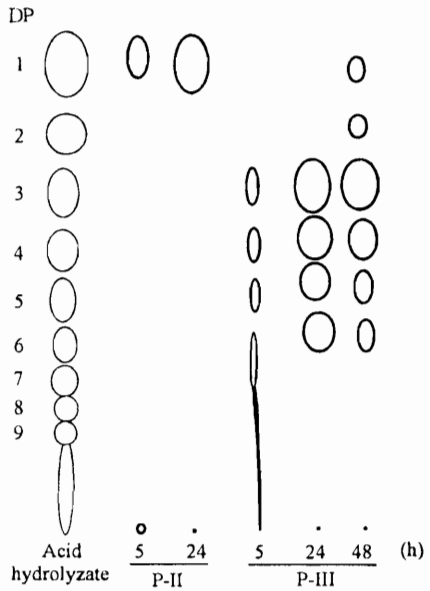

Fig. 2. Paper chromatograms of inulin hydrolyzates by inulinases P-II and P-III from *A. niger* 12.

products after 24 h of incubation. Small amounts of fructose, sucrose, and inulobiose began to appear in the reaction mixture after 48 h of digestion.

Time courses of inulin hydrolysis by intracellular inulinases

The time courses of inulin hydrolysis by intracellular inulinases P-II and P-III are shown in Fig. 3A. An inulinase P-II hydrolyzed inulin completely in 24 h. Inulinase P-III displayed fast hydrolysis of inulin during the first 3h of incubation, and then gradually hydrolyzed inulin to the extent of 47 % after 24 h. The liberation of free fructose and glucose from inulin by exoinulinase P-II was determined (Fig. 3B). The high ratio of total hexose to glucose during the first 12 h showed that the exoinulinase P-II starts hydrolysis of inulin from the fructose end.

Action of endoinulinase P-III on various oligosaccharides

The reaction mixture, consisting of 0.5 ml of each substrate (10 mM) in 0.1 M acetate buffer (pH 5.0) and an equal volume of the enzyme solution (1.0 U/ml) in the same buffer, was incubated at 40 ℃ for 48 h, and it was sub-

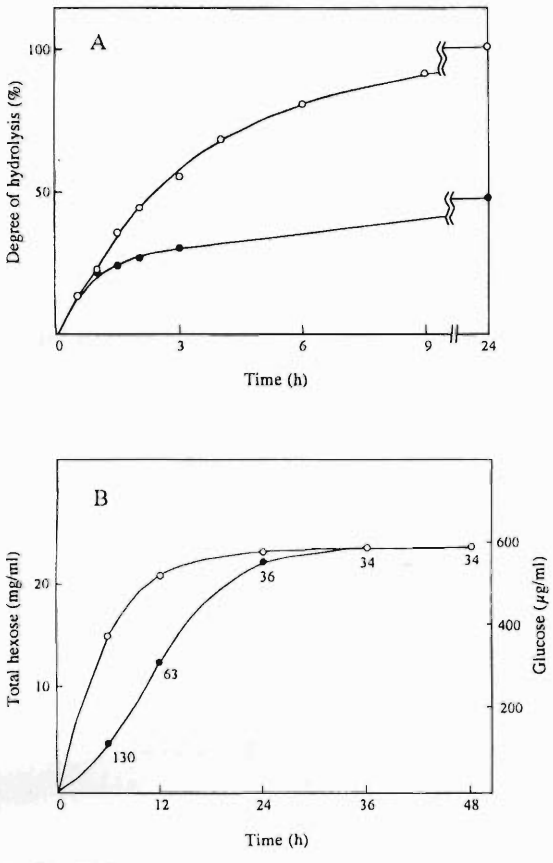

Fig. 3. Action pattern of inulinases P-II and P-III from *A. niger* 12

(A) Time course of inulin hydrolysis by inulinases P-II and P-III. Symbols: ○, P-II; ●, P-III. (B) Total hexose and glucose contents during enzymatic hydrolysis of inulin. The numbers at each point on the glucose curve represent the ratios of total hexose to glucose. Symbols: ○, total hexose; ●, glucose.

jected to TLC (Fig. 4). TLC showed that the endoinulinase P-III did not act on sucrose, inulobiose (F_2), or 1-kestose (GF_2). The enzyme weakly hydrolyzed F_3 to F_2 and F, GF_3 to F_3 and G, F_4 to F_3 and F, and GF_4 to F_3 and GF.

Discussion

Snyder and Phaff[10] showed that the intra- and extracellular inulinases of *S. fragilis* (currently known as *Kluyveromyces marxianus* var. *marxianus*) were identical with respect to substrate specificity, rate of inactivation by heat, pH optima for inulinase and invertase activities, and elution patterns of column chromatographies. Subsequently, Negoro[11] showed that intra- and extracellular inulinases from *K. fragilis* (currently *K. marxianus* var. *marxianus*) had similar enzymatic properties except that the intracellular enzyme was more labile to urea, guanidine hydrochloride, and SDS than the extracellular one. An intracellular exoinulinase P-II with M_r of 47 kDa and I/S ratio of 0.30 from *A. niger* 12 in the present study was distinct from two forms of extracellular exoinulinases P-I (72 kDa)[3] and P-II (59 kDa)[4] that show I/S ratios of 0.23 and 0.80, respectively. Intra- and

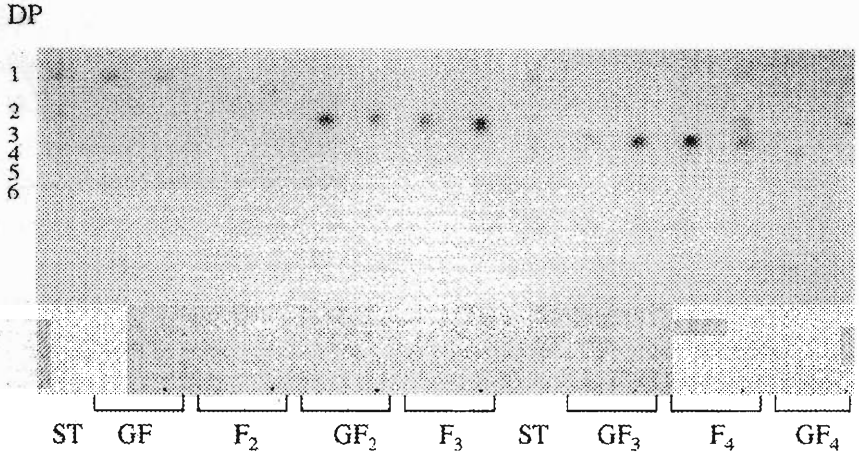

Fig. 4. TLC of the hydrolysis products of various oligosaccharides by endoinulinase P-III from *A. niger* 12.

Fructo-oligosaccharides (GF_n) and inulo-oligosaccharides (F_m) were used as substrates for endoinulinase P-III (the subscript *n* or *m* stands for the number of fructosyl moieties). Substrates and reaction products are shown on left and right sides of each pair, respectively.

extracellular endoinulinases from *A. niger* 12 differed in electrophoretic mobility, thermal stability, and K_m value among the properties investigated. A larger intracellular endoinulinase P-III (56 kDa) was more stable up to 60 ℃ as compared to 50 ℃ in the smaller extracellular enzyme (54 kDa)[5]. The K_m value of 0.80 mM for the intracellular endoinulinase P-III was lower than the reported value of 1.25 mM for the extracellular equivalent. Improved properties of the intracellular endoinulinase, including thermal stability and affinity for inulin, might be ascribed to the structure or content of the carbohydrate moiety of the protein.

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