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Difructose Anhydride-Forming Bacterial Inulinase II and Fructogenic Fungal Inulinase I

Free and Immobilized Forms

MADALENA BARON,*[,1] JOSÉ A. FLORÊNCIO,[1] GISSELA M. ZANIN,[2] ANTONIO G. FERREIRA,[3] ROGERIO ENNES,[1] AND JOSÉ D. FONTANA[1]

[1]Biomass Chemo/Biotechnology Laboratory, Department of Biochemistry, Federal University of Paraná (LQBB-VFPR), PO Box 19046 (81.531-970), Curitiba, PR, Brazil; [2]Department of Chemical Engineering, State University of Maringá, Maringá, PR; and [3]RNM Laboratory, Chemistry Department, Federal University of Sao Carlos, Sao Carlos, SP

ABSTRACT

The reserve polymeric inulin from dahlia tuber (>12% or >60% yield, wet or dry basis, respectively) follows as an attractive source for both free fructose or difructofuranose anhydride (DFA III). Although DFA biological activity is not completely understood, there is interest in characterizing other DFA III-producers besides *Arthrobacter ureafaciens*. The inulinolytic bacterial isolate named "YLW," owing to the yellow hue in agar slants, is such a producer. Its biochemical characterization showed the presence of galactosylated and mannosylated glycolipids associated with the bacterial cells. Immobilization of fungal inulinase I and bacterial inulinase II, the respective enzymatic catalysts for the production of fructose and DFA III by inulin hydrolysis, was attempted using controlled-pore silica (CPS). The effects of pH, temperature, and incubation time was analyzed and compared for both enzymes in the free and immobilized forms.

Index Entries: Inulinases I and II; "YLW" bacterial strain; glycolipids; fructose and DFA III (difructose anhydride); immobilization.

INTRODUCTION

The outstanding position of fructose within the world sweetener market is explained by its natural occurrence, well-established biotechnological production from starch, superior degree of sweetness and noninsulinogenic behavior. (β-[2 → 1'], α-[2' → 3]-difructofuranose anhydride) (DFA III), a natural but rare anhydro form of inulobiose *(1)*, also deserves attention owing to the interference on the in vivo-free fructose metabolism and its less polar structure. These properties might support future prospects, such as dietetic use and as a chemical basis for novel procedures of glycoderivatization *(2)*. Hence, the competitive routes for generation

*Author to whom all correspondence and reprint requests should be addressed.

of fructose and DFA III from inulin (a plant reserve poly-β-2,1'-fructofuranosic polymer) could be explored through immobilization of the respective biological catalysts: fungal type I inulinase and bacterial type II inulinase. Microbial inulinases have been extensively considered *(3)* and appointed as interesting biotechnological devices. Our option for the second enzyme model is a bacterial isolate *(4)* previously named "YLW" (from its yellow hue in agar slants), which displayed a similar, but not identical random amplification of polymorphic DNA-polymerase chain reaction (RAPD-PCR) profile as compared to that for *Arthrobacter ureafaciens (5),* the pioneer source for the DFA III. Controlled-pore glass (CPS) was thus selected to launch the specific objective of immobilization trials of the inulinolytic enzymes. A commercial preparation of fungal inulinase I (*Aspergillus ficuum;* NOVOZYM 230, Novo Nordisk, Curitiba, Panama, Brazil), owing to the ease of provision, is being used as a reference model for this scope, and our accumulating experience will be further extrapolated to inulinase II from the YLW bacterial isolate. An excellent result on *Aureobasidium* sp. (dimorphic yeast) fructofuranosidase immobilization has recently been reported *(6).*

Inulin, the compulsory substrate, may be easily prepared from dahlia tubers using organosolvent precipitation followed by anionic exchange on DEAE-cellulose *(1,4).*

Since the local inulinolytic isolate designated YLW proved to be a superior DFA producer, its biochemical characterization is also in progress. For this, the occurrence of two differentiated and cell-associated glycolipids is reported.

MATERIALS AND METHODS

Microorganism and Enzyme Sources

The bacterial YLW isolate was previously isolated from *Dahlia pinnata* rotting tubers *(1,4).* Liquid cultures (400 mL) with the YLW bacterium were carried out in 2-L Erlenmeyer flasks with an air:medium ratio 5:1 at 100 rpm, at 28°C for 5 d, after successive cell transfers from agar slant to 25– (125–mL flask) and 100-mL (500-mL flask) inoculating cultures. Media for scaled-up liquid cultures received 3 g% inulin and 0.6 g% yeast extract, reinforcement being made with 0.1 g%, pH 6.0, ammonium dihydrogen phosphate and 0.08 g% magnesium sulfate. Cells were harvested by centrifugation at 8000 rpm for 30 min at 5°C, and the cell pad processed as further described. The supernatant, which displayed pink-wine color, was extensively dialyzed against distilled water at 4°C, and then lyophilized. The resulting sticky dried and heavily dark-colored material was used as source of type II bacterial inulinase.

A commercial product (Novozym 230, ex-*A. ficuum*) received as gift from Nova Nordisk was used as active source of type I fungal inulinase. Protein contents were estimated with the biuret reagent or with Bradford's reagent *(7).*

Enzyme Activity Assay

Routine incubations of inulinases I and II were carried out in a final volume of 1 mL containing 0.5 mL of prewarmed 6 g% inulin, 0.1 mL of 0.5*M*, pH 4.7, sodium acetate or 0.1 mL of 0.5*M*, pH 5.5, citrate buffers, respectively, and 100–250 μg of total solids as enzyme(s) source. Agitation (150 strokes/min) was minimally provided in a Cole Parmer bath (Chicago, IL). The reaction at 50°C was usually stopped at 1 or

3 h by the addition of alkaline dinitrosalicylate (in case of reducing sugar) or 3 vol of iced methanol (in case of chromatographic analyses). Fructose (as reducing sugar) and DFA III (nonreducing modified disaccharide) were qualitatively monitored by thin-layer chromatography (TLC) on silica gel 60 chromatoplates (Merck, Darmstadt, Germany) with mobile phase of isopropanol:nitromethane:ethyl acetate:methylethylketone:methanol:water (50:45:50:25:10:20). Anisaldehyde and orcinol, both prepared in 0.5 g% methanolic solution containing 5% (v/v) sulfuric acid, were used as general and specific (glycocompounds) revelators, respectively. Quantitation of fructose and DFA III was performed with high-pressure liquid chromatography (HPLC) in a Waters SugarPak column irrigated with water at 85°C (0.5 mL/min; 450–460 psi) using a Waters multimodule assembly (SC 600E/Wisp 712 with a 410 DR recorder). Fructose, as reducing sugar, was also measured with the alkaline dinitrosalicylate reagent.

Enzyme Isoelectrofocusing

A concentrated solution of inulinase II (up to 200 mg solids/1 mL), partially purified by ion-exchange chromatography on DEAE-cellulose using 0.1*M* sodium acetate, was spotted onto a Pharmacia Ampholine 3.5–9.5 PAGplate and run for 6–8 h with increasing voltage input from 100 to 1000 V (not exceeding 10 mA and/or 2 W). Routine standards were 3.5–9.5 pI kit of proteins (Pharmacia, Uppsala, Sweden) and anionic microheterogeneous L-amino acid oxidases from snake venoms *(8)*. Gel plates were fixed with 28 g% trichloroacetic acid (TCA) containing 7.5 g% of sulfosalicylic acid and stained with the usual formulation of Coomassie brilliant blue.

Enzyme Immobilization Trials

An already published protocol, with minor modifications, was adopted for the scope of inulinase immobilization on controlled-pore silica (CPS) *(9,10)*. The CPS particle size was 0.5 mm (mesh size 30/45) containing pores of 375 Å. After treatment of the CPS beads with 3-aminopropyltriethoxysilane, the silica was activated using 2.5% glutaraldehyde solution. The ratio of enzyme input (as nonpurified total solids; dry basis) to support was variable (1:10, in case of inulinase I on CPS). The activity of free and immobilized enzyme(s) was preliminarily evaluated for pH, temperature, and incubation time effects.

Glycolipid Extraction, Purification, and Sugar-Moiety Analysis

Lyophilized YLW cells (either from inulin- or starch-based *(11)* culture media) were sequentially extracted with hexane and chloroform (triglycerides elution), ethyl acetate, acetone, ethanol, and finally methanol (polar and charged lipids). Fractions containing orcinol (+) compounds were pulled and applied in a silicic acid column for glycolipid enrichment or purification using a gradient of acetone in chloroform.

RESULTS AND DISCUSSION

Glycolipids may be found in Arthrobacteriaceae, a type II inulinase-producing family. One example for them is that resulting from the interspersing of sugar and hydroxy-fatty acid units through glycosidic and ester linkages *(11)*, which

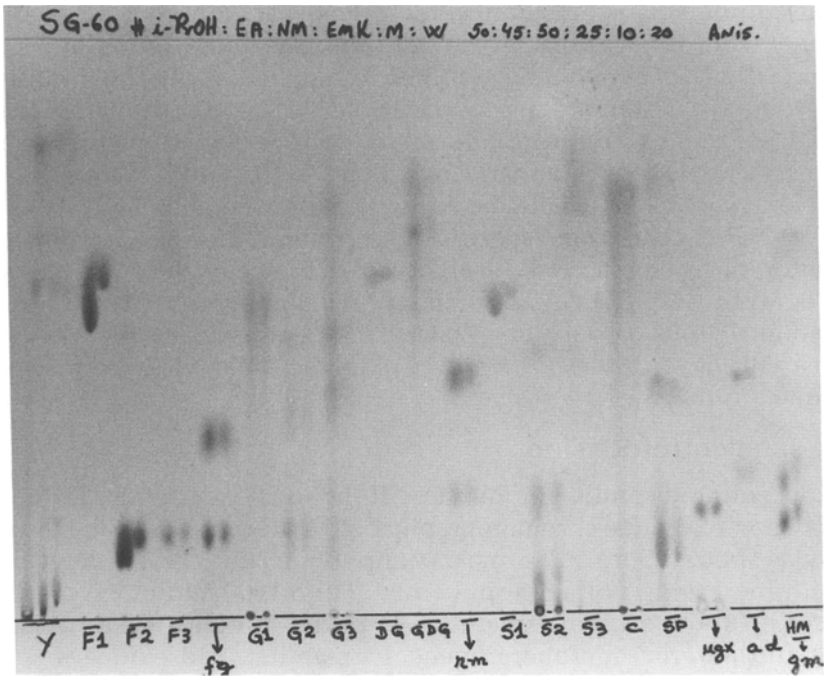

Fig. 1. TLC of the YLW bacterial cell-associated glycolipids. Samples: Y= crude lipid extract from YLW cells (including the fast and slow glycolipids in the Rf, 0. 6–0.7 zone); F1, S1, and G1, fast and slow purified YLW glycolipids and a bovine galactosylceramide standard, respectively; F2, S2, and G2, hydrolysis products from F1, S1, and G1, partition aqueous phase; F3, S3, and G3, hydrolysis products from F1, S1, and G1, partition organosolvent phase. Standard: DG, dodecylglucoside; GDG, galactosyldiglyceride; C, SP, and HM, ceramide, sphingosine, and hydroxymyristic acid; fg, L-fucose and D-galactose; rm, L-rhamnose and D-mannose; ugx, D-glucuronic acid, D-glucose, D-xylose; gm, D-galactose and D-mannose; ad, L-arabinose and DFA III.

have weak inhibitory properties against HeLa cells. Other microbial glycolipids are glycosilglycerides. It was found that YLW strain whole-lipid extract contained a significant amount of two related glycolipids obtained as enriched preparations when fluting the silicic acid column with chloroform:acetone (3:2 → 2:3; "fast" glycolipid) and pure acetone ("slow" glycolipid). Their chromatographic nobilities were above and below, respectively, that for the dodecyl-glucose standard (Fig. 1; lanes Y and DG). Furthermore, they were intensively stained by the chromogenic reagents orcinol (sugars) or *p*-anisaldehyde (general lipid compounds) with different hues in the second reagent. Given our interest in a deeper biochemical characterization of this DFA III-producer bacterial strain, the purified glycolipids (designated fast and slow, respectively; lanes F1 and S1, Fig. 1) were submitted to acid hydrolysis with trifluoroacetic acid (1.3*M* TFA; 100°C; 3 h) and each hydrolysate partitioned among chloroform: methanol:water (48:48:4) in order to recover separately the carbohydrate and lipid moieties. The same acid fragmentation procedure was applied to a reference model, that is, bovine galactosylceramide. The correspondent upper (lanes F2, S2, and G2; Fig. 1) and lower phases (F3, S3, and G3; Fig. 1) of partition were included in the TLC along with some strategic sugar standard (for

instance, lanes fg = fucose + galactose; rm = rhamnose + mannose). The TLC indicated that galactose and mannose were the respective carbohydrate moieties of "fast" and "slow" YLW glycolipids. A ^{13}C nuclear magnetic resonance (NMR) run for the intact "fast" galactosylated glycolipid (the higher purified sample; spectrum not shown) confirmed the occurrence of linked C1 (δ = 103.9 ppm) and free/esterified C6 (δ = 61.76 and 62.89 ppm) of β-D-galactopyranosyl unit. Carbon-1 of free and *O*-linked β-galactopyranosyl units usually presents chemical shifts in δ 97.7 and δ 103.7/105.8 ppm, respectively *(12,13)*. The presence of nine signals of relative similar intensity in the δ = 110 → 60 ppm spectral region was suggestive of the combination of galactose (6C) and glycerol (3C), and then the possibility for occurrence of a (mono- or di-)galactosyldiglyceride (standard at lane GDG, Fig. 1). Also present, at the lowest magnetic field, are two clear signals at δ = 173.96 and δ 173.58 ppm; these are characteristic for C = O from ester linkages *(11)*. Given the occurrence of both galacto- and mannolipids in the YLW strain, the fine chemical structure of each lipoglycoconjugate remains to be established. Between 50 and 100 different lipid structures are found in cell membrane *(14)*.

Protein population analysis of the inulinase sources proved to be a hard task. The fungal commercial preparation, counterbalancing its high inulinase I (fructogenic) activity, is a dark brown viscous syrup with a content in total solids >280 mg/mL, mostly recovered when attempting to enrich the protein population through precipitation with 20% TCA or 3 vol of iced ethanol. These fractions, as well as the crude enzymatic preparation, carried substances that interfered with the usual techniques for protein quantitation (biuret, Folin-Ciocalteu, or Bradford's Coomassie). The same problem was faced with the YLW inulinase II preparation, a deeper dark-colored solution with a similar total solid content/mL. In an investigation of the complex of ends- and exoinulinases from *A. ficuum (15)*, it was demonstrated that the presence of eight isoenzymes purified by ion-exchange chromatography and their glycosylation degree may reach up to 41%. For the inulinase II preparation, the protein population was analyzed by electrophoretic procedures that were negatively affected owing to the need of sample overload to ensure a minimum amount of visible protein with the sensitive Coomassie staining. The polyacrylamide gel isoelectrofocusing of a partially purified inulinase II fraction, using a large excess of sample load (Fig. 2, lane Y), indicated only three well-marked protein bands whose pI values were in the range between 3.50 (amyloglucosidase) and 4.55 (soybean trypsin inhibitor). It is not known yet if the inulinase II fine structure is also a glycoprotein, so the glycomoiety is not revealed by the Coomassie staining. Such a fact can explain the difficulty in visualizing bands of protein in the inulinase II gel. In our experience, the yeast invertase is an illustrative case owing to its huge carbohydrate content (>50%).

The first batch of immobilized enzyme was made available (in case of inulinase I on CPS) and the linkage of protein–enzyme to the support was confirmed with Coomassie staining and optical microscopy of the blue-stained particles. The effect of kinetic parameters for free and immobilized enzymes was then measured and the results comparatively shown in Figs. 3 (incubation time) and 4 (temperature). The immobilized enzyme performed approx 80% of inulin monomerization to reducing sugar as fructose, when compared with the hydrolysis by free enzyme in long-term incubation (Figs. 3A and B). Furthermore, it was observed that half of the substrate conversion was attained with the free enzyme in a shorter time (25 min). The longer time seen with the CPS-immobilized enzyme for the same purpose

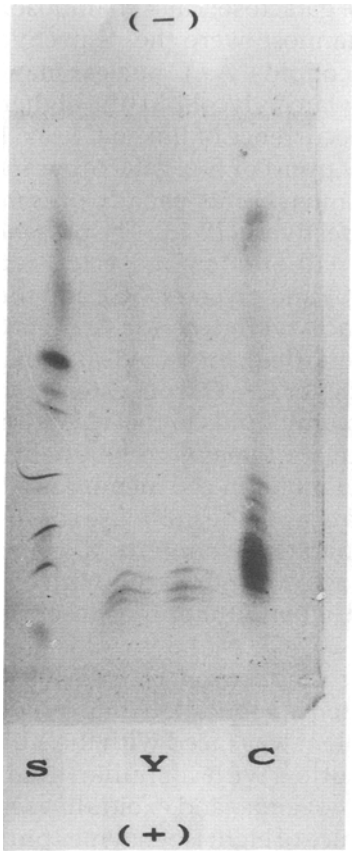

Fig. 2. PAGIF—polyacrylamicle gel isoelectrofocusing of the bacterial (lane Y) inulinase II. Standard: Ca, *Crotalus atrox* anionic L-amino acid oxidases; pI, isoelectric focusing standard kit (pI, 3.5–9.5).

(60 min) may be explained by limitation of the substrate diffusion. The optimal 50–60°C temperature range for the free enzyme (Fig. 4A) moved sharply to 50°C (Fig. 4B) as a result of the immobilization procedure. A similar effect was found when immobilizing inulinase I from *Fusarium oxysporum* on AE-cellulose with glutaraldehyde *(16)*. The same situation was observed for the pH effect because the optimum pH range around 4.5 seen for the free enzyme was displaced toward a more acidic zone (Fig. 5) when using CPS-immobilized enzyme.

For the second enzymatic model, the effect of the temperature and pH on bacterial inulinase II (free enzyme) activity, which converts inulin to DFA III, is shown in Figs. 6 and 7, respectively. The optimum temperature for free inulinase II was at 50°C. Concerning to the pH effect, the curve displayed two peaks in pH around 5.5 and 7.0. Since the Ca^{2+} ions (up to 10–100 m*M* range) were revealed to be beneficial for free fungal inulinase I (up to 40% activation), it is also advisable to verify the effect of divalent cations on free and immobilized bacterial inulinase II activity, and work is in progress as part of the immobilization procedures for this enzyme. A recent report demonstrated that yeast inulinase is activated by several

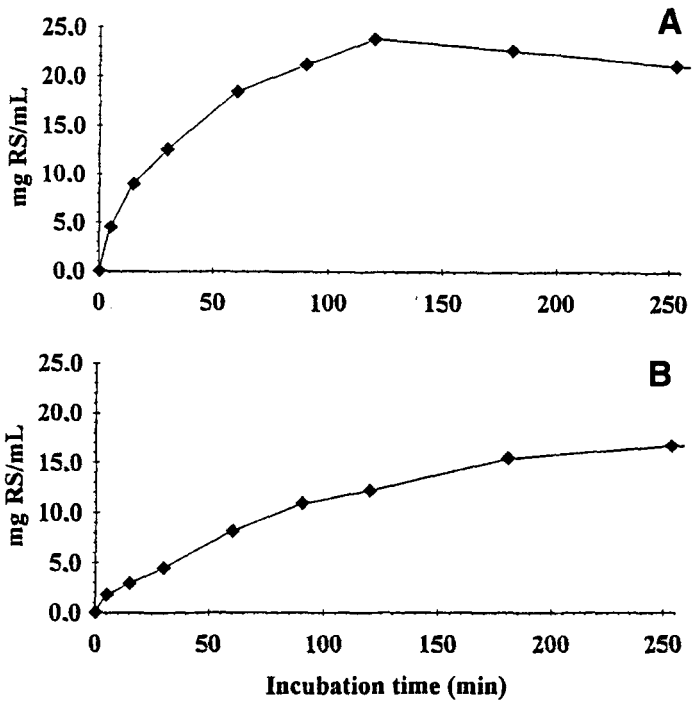

Fig. 3. Incubation time monitoring for the activity of the free **(A)** and CPS-immobilized **(B)** fungal inulinase I. RS, reducing sugar (as fructose).

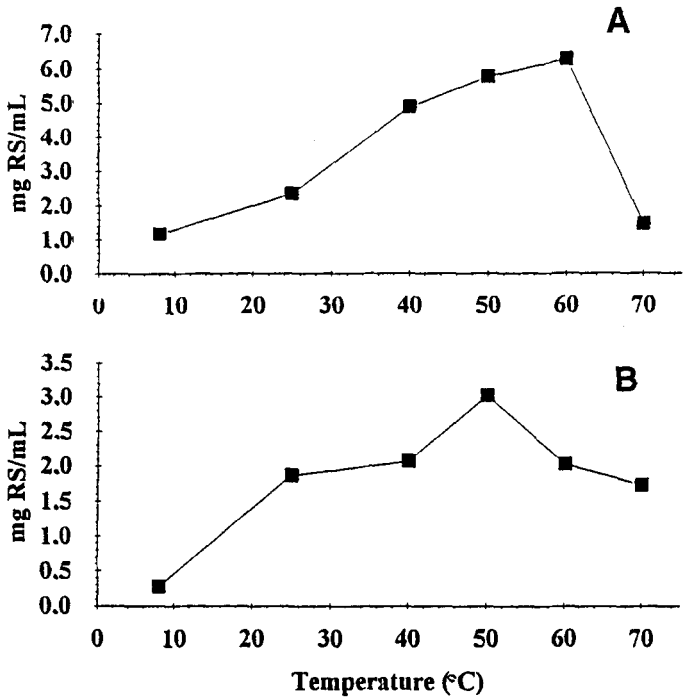

Fig. 4. Effect of the temperature on the hydrolytic activity of the free **(A)** or CPS-immobilized **(B)** fungal inulinase I. RS, reducing sugar (as fructose).

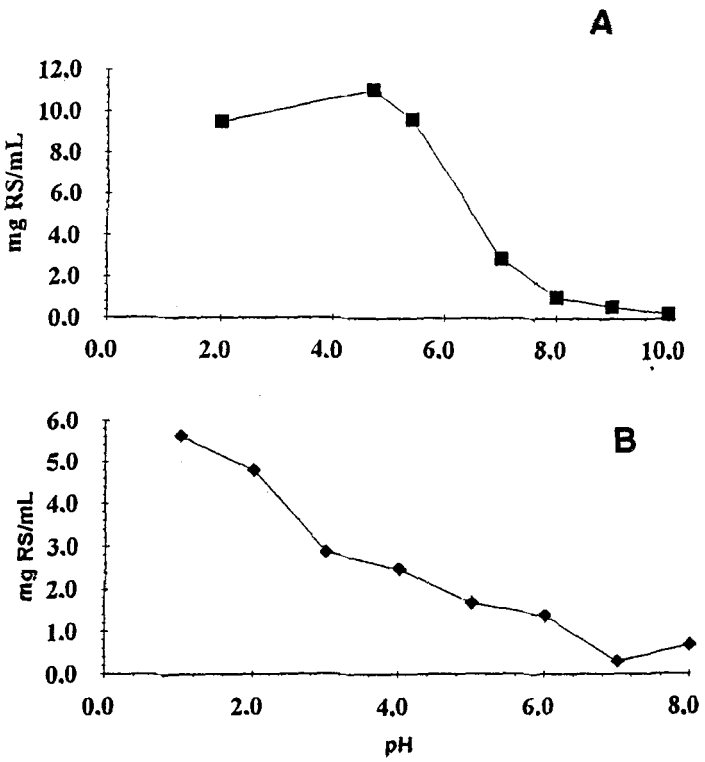

Fig. 5. Effect of the pH on the hydrolytic activity of the free **(A)** or CPS-immobilized **(B)** fungal inulinase I. RS, reducing sugar (as fructose).

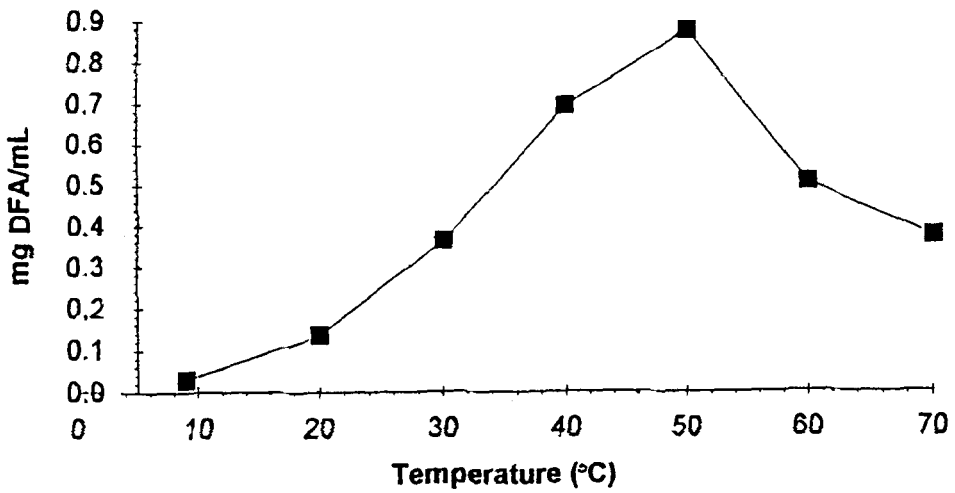

Fig. 6. Effect of the temperature on the hydrolytic activity of the free bacterial inulinase II.

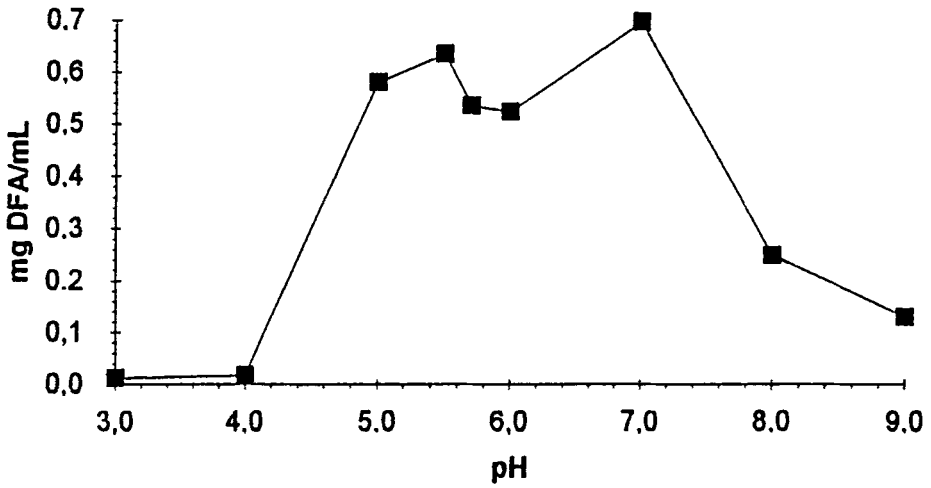

Fig. 7. Effect of the pH on the hydrolytic activity of the free bacterial inulinase II.

divalent cations, except Hg^{2+}; the concentration of 1 m*M* Ca^{2+}, for instance, resulted in a 33% activity increase *(17)*.

Among the methods for enzyme activity monitoring, HPLC (quantitative) and TLC (qualitative) proved to be very useful. When analyzing sugar standard (DFA and fructose; Fig. 8A) and reagent controls (residual inulin and methanol as precipitating solvent; Fig. 8D), HPLC was a fast and high resolution method (10–15 min for each full analysis). The activity profile for free (Figs. 8B and E) and CPS-immobilized (Figs. 8C and F) inulinases I and II was also recorded, showing the production of fructose and DFA III, respectively, from inulin. Although both enzymatic solutions were prepared with the same total solid content/mL, the performance of the bacterial enzyme was lower than that of the fungal inulinase I, since the first enzyme showed the product DFA III and also nonreacted inulin in the HPLC analysis. In the case of the second enzyme, thes inulin substrate was almost completely consumed in the reaction. Not only for the immobilization scope itself, but also for attribution of specific activity values, both inulinase I and II preparations are being purified through gel filtration and anion-exchange chromatographies. In this way, the immobilization procedures of crude inulinase preparations and their purified fractions will continue to deserve our attention when using CPS, which showed feasibility for this purpose. The use of organic supports also will be tested in ongoing work.

CONCLUSIONS

The biochemical knowledge about an inulinolytic bacterial isolate (YLW) proceeded with the partial characterization of two cell-associated glycolipids whose carbohydrate moieties are galactose and mannose.

Immobilization of fungal inulinase I (fructogenic) and bacterial (YLW) inulinase II (difructose anhydride producer) was carried out using CPS, which was shown to be a suitable support for immobilizing both enzymes.

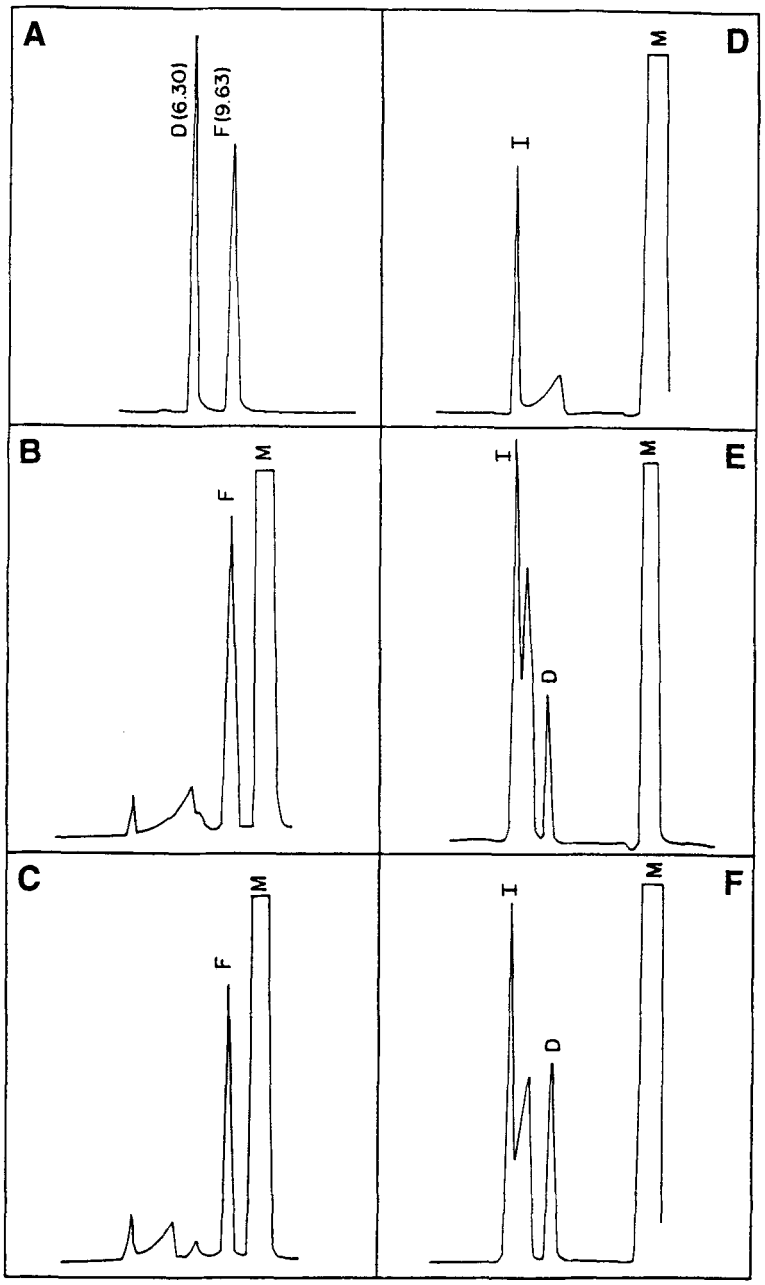

Fig. 8. HPLC analyses of the comparative performance of free and CPS-immobilized forms of inulinases I and II. A, DFA III and D-fructose standard; D, substrate control (buffered inulin precipitated with methanol; analysis of the supernatant); B, C, free and immobilized fructogenic fungal inulinase I; E, F, free and immobilized DFA-producer bacterial inulinase II (M, methanol; I, residual inulin; the unlabeled peaks arose from acetate or citrate-buffering salts).

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