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Microbial Inulinase Secretion Using Chemically Modified Inulins

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ABSTRACT

Caproyl and cholesteryl derivatives of native dahlia inulin were prepared from the respective chloride donors, and the light derivatization was monitored by ^{13}C-NMR and by FTIR. These inulin derivatives were employed as carbon sources and as inulinase inducers using different strains of the inulinolytic yeast *Kluyveromyces marxianus*.

A low but consistent basal inulinase activity (constitutive) was expressed in control cultures grown in carbon source-free and yeast extract-based media, independently of the salt supplement. A higher enzyme activity resulted from induction with native inulin. The highest inulinase activity, in U/mL of cell-free medium, was attained with the lipophilic inulin derivatives.

Caproylated inulin was superior as an inulinase inducer compared to the cholesteryl derivative. The native inulin-based medium was used as reference for the induction of inulinase in the IZ-275 yeast strain at 28°C and 100 rpm for 70 h. With caproyl-inulin, a 6.8-or 4.9-fold increase of the units of inulinase/mL of cell-free medium was observed, respectively, in the absence or presence of ammonium phosphate supplement. The corresponding values for the IZ-619 yeast strain were 4.9 and 1.8. Cholesteryl-inulin did not induce the IZ-265 strain, but IZ-619 inulinase activity experienced a 4.1-fold increase in the ammonium phosphate supplemented medium. Thus, the induction/secretion process of inulinase is affected by the presence of ammonium phosphate, depending on the yeast strain and the modified inducer.

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Caproyl-inulin also stimulated the growth of an inulinolytic bacterium isolated from rotting dahlias and its bioconversion of inulin to DFA III, a difructose anhydride.

Index Entries: Caproyl-inulin; cholesteryl-inulin; inulinase induction.

INTRODUCTION

Fructans, which are short polymers of β-1,2-linked D-fructose and reserve carbohydrates, occurring in roots and tubers of several *Compositae*, represent convenient sources for the preparation of alternative high fructose syrups (HFS). Syrups arising from corn starch enzymatic monomerization and partial isomerization (HFCS), are still dominant in the sweetener market (*1*). The noninsulinogenic effect of D-fructose, as compared to glucose, and more importantly, its 75% enhancement as a sweetener as compared to sucrose (*2*), account for the wide acceptance of HFCS as preferential sweeteners. Two advantages of *Dahlia* inulin as a raw material for fructose production are the high tuber inulin content (>50%, dry wt basis) and the high fructose:glucose ratio of the native polymer, ~30–35:1, owing to the single sucrosyl unit at one of the nonreducing ends of the linear polysaccharide chain. The extreme acid susceptibility of inulin is another advantage in the case of nonenzymatic processing. Recently, mild acid hydrolysis using thermopressurization of an inulin paste with dilute phosphoric acid (acidification to pH 3.4) was carried with the object of complete fructan monomerization combined with concomitant absence of by-products such as hydroxymethylfurfural (HMF) and/or difructose anhydrides (DFA) (*3*). The use of mold and yeast endo and/or exo-inulinases are alternatives, but enzymatic inulinolysis to fructose would only be feasible if the required enzymes were to be available in large quantities at a competitive price. For this, genetically engineered inulinolytic microorganisms and the biochemical engineering of already known and available wild-type yeast are valid alternatives.

In contrast to baker's yeast invertase, inulinases are nonspecific β-fructofuranosidases able to hydrolize both (2→1′)- and (2→6′)-linked fructans of various chain lengths as well as sucrose. Hence they could be used as a replacement for invertase in the confectionary industry. Yeast inulinases have been characterized as secretory glycoproteins in a number of yeasts, *Kluyveromyces marxianus* being the classic source (*4*).

A drawback of enzymatic hydrolysis of inulin is its very low solubility in water at room temperature. However, two improvements in enzymatic depolymerization can be effected by insertion of acyl or condensed ring substituents into inulin, resulting in: change in the solubility of the substrate/inducer, and/or facilitate contact of the substrate/inducer with the

inulinolytic microorganism cell envelope, which positively affects the inulinase induction process. Considering these possibilities, we decided to derivatize dry purified native *Dahlia* inulin with caproic acid or cholesterol using their respective chlorides as reagents. Strains of *Kluyveromyces marxianus* were selected as target inulinolytic microorganisms to evaluate the desired effects of inulin derivatives.

MATERIALS AND METHODS

Preparation of Inulin and Their Derivatives

Inulin was obtained from *Dahlia* tubers (3) and further purified by filtration of a warm solution through a column of DEAE-cellulose (acetate form) to remove all acidic contaminants and a yellow pigment. The aqueous filtrate was added to 3 vol of absolute ethanol and the resulting insoluble paste lyophilized.

Caproyl-inulin (Capr-In) was prepared by reaction of equimilimolar amounts of moisture-free inulin (anhydrofructose basis) and caproyl chloride. The reaction solvent was a 1:2 (v/v) mixture of dimethylsulfoxide and pyridine (previously dried with molecular sieves) containing sodium carbonate in an amount of 10% of the combined inulin and C-6 acyl donor. The solvent container (a rotaevaporator flask) was kept in an ice bath during the addition of reagents and then vacuum-applied. Occasional addition of crushed ice to the rotaevaporator bath ensured a controlled temperature at ~8°C for 30 min. The reaction mixture was centrifuged to remove residual carbonate and resulting sodium chloride, and the caproylated inulin recovered from the supernatant by addition of 2 vol of ethanol followed by 2 vol of isopropanol. The precipitate, after high vacuum, resulted in a water-soluble, yellowish powder.

Cholesteryl-inulin (Chol-In) was synthesized under similar conditions, except that: the cholesteryl chloride:inulin ratio was 10:3; carbonated pyridine was used as solvent and HCl-neutralizing agent; and room temperature incubation was for a longer period (24 h). Excess ice-water was added to the clarified reaction supernatant, and the precipitated cholesteryl-inulin lyophilized, which also resulted in a yellowish powder with a reduced solubility in water.

^{13}C-Nuclear Magnetic Resonance (NMR) Spectroscopy

The ^{13}C-NMR spectra of the native inulin and of its caproyl derivative were recorded with a Bruker AC-300-P Spectrometer using DMSO-d_6 solutions. The spectral width was 19.2 KHz, the acquisition time 0.85 s, the pulse width 21 μs, and the number of transients up to 60,000 depending on the sample size. The temperatures for the spectral runs of inulin and its caproylated derivative were 70°C and 25°C, respectively.

Fourier Transform Infrared (FTIR) Spectroscopy

Infrared spectra, Fourier transform mode, of the native inulin and of its cholesteryl derivative were obtained using a model 1600 FTIR Perkin-Elmer apparatus, using milligram amounts of inulin or derivative in a KBr disk.

Inulinolytic Microorganisms and Culture Conditions

Kluyveromyces marxianus var. *marxianus* IZ-275 and IZ-619 were provided by P. Vertoni (Department of Science and Technology, ESALQ-USP, Piracicaba, Sao Paulo). These yeasts were maintained on agar slopes containing 1 g% fructose, 0.1 g% ammonium dihydrogen phosphate, and 0.2 g% yeast extract. Agitated liquid medium culture was at 28°C and 110 rpm using 0.2 g% yeast extract plus 0.5 g% inulin inducer/substrate or their derivatives and supplementing or not the basic medium with 0.1 g% ammonium dihydrogen phosphate. The ratio of air to medium volume in the Erlenmeyer flasks was 5:1.

As a bacterial model for the evaluation of the physiological effect(s) of inulin derivatives, the isolate "YLW" (owing to the colony yellow color) from rotting *Dahlia* tubers was used (*3*). The inulin or inulin derivative concentration in the culture medium was increased to 2 g%, the ammonium phosphate suppressed, and yeast extract supplementation was maintained as 1/10 of the carbon source/inducer. The light inoculum corresponded to a starting absorbance about 0.010–0.020 at 650 nm. The resulting difructose anhydride (DFA III) was determined by HPLC (*3*).

Liquid culture for fructose- and DFA-producing inulinases assays was sampled (1 mL) at each 12- or 24-h intervals. Cell mass and cell-free culture fluid were separated and maintained at 4°C until analysis.

Inulinase Assay

Enzyme activity assay was based on the release of reducing sugar from a prewarmed solution of 2% (w/v) purified inulin. The culture fluid (0.1 mL) was incubated at 50°C with the substrate in the presence of 0.1*M* pH 5.0 sodium acetate buffer (0.4 mL) for 10–30 min, depending on the enzyme activity level. The released free sugars were analyzed using the alkaline dinitrosalicylate (DNS) reagent (*5*). Inulinase(s) activity was expressed as μmol of fructose$\cdot h^{-1}\cdot mL^{-1}$ of cell-free medium.

RESULTS AND DISCUSSION

The ^{13}C-NMR spectra of our *Dahlia* native inulin preparation (Fig. 1A), following purification by ethanol precipitation and anionic exchange resin treatment, gave a clear indication that the desired polysaccharide purity was attained. The observed signals are in accordance with those expected from the fructose carbons, being at δ in ppm (*6*): C-1 (61.6), C-2 (103.1),

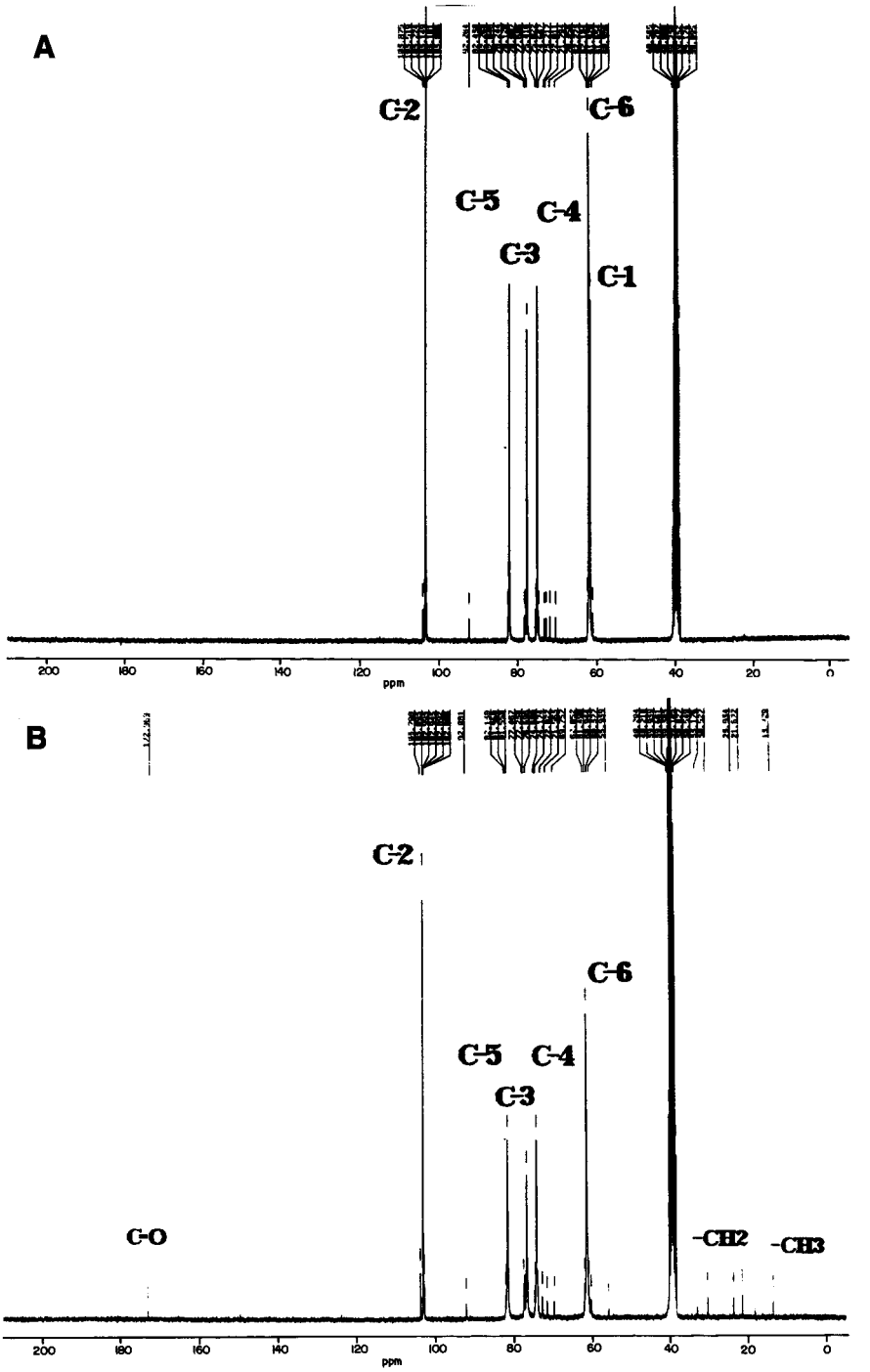

Fig. 1. (**A**) ^{13}C-NMR spectrum of purified native inulin from *Dahlia* tubers. (**B**) ^{13}C-NMR spectrum of caproyl-inulin. (C-1 to C-6 indicated the carbon numbers of the anhydrofructofuranose units of the polymer as the sources of the main spectral signals; -C=O, CH_2, and -CH_3 indicated the secondary spectral signals arising from the chemical groups of the caproyl substituent; the largest peak of δ 40 ppm is the DMSO-d_6 reference).

C-3 (77.6), C-4 (75.0), C-5 (82.0), and C-6 (61.9). As a useful indication of inulin molecule integrity, a group of low intensity signals was evident, arising from the glucose moiety of the single sucrosyl unit at one of the two nonreducing end units of inulin, being at δ in ppm: C-1 (92.2), C-2 (71.8), C-3 (73.2), C-4 (70.3), C-5 (72.8), and C-6 (61.6). The signals at δ = 103.1 and 92.2 ppm of the C-2-β-fructofuranose and C-1-α-glucopyranose rings, respectively, are typical of inulin spectra. The spectra of inulin derivatized with caproyl residues (Fig. 1B) resulted in the following modifications: first, additional and somewhat distinct signals at higher field (δ = 13.7 to 33.1), corresponding to the $-CH_3$ and $-CH_2$-groups of the caproyl residue with one extra signal at lower field (δ = 172.9), typical of the carbonyl group of the ester linkage; and second, a final feature was the modification of prominent doublet of native inulin (C-6 and C-1; δ = 61.9 and 61.6), which, in the caproyl derivative, appeared as a broad single signal. This agrees with the expected substitution of native inulin primary alcohol groups namely C-6 and C-1, by caproyl residues. Also importantly, the light derivatized (caproyl)-inulin, advantageously, was soluble in water at room temperature, as desired.

Some characteristic frequencies of infrared absorption by native inulin chemical groups is shown in Fig. 2A as cm^{-1} (7), the broad band at 3200–3500 from O-H stretching, the C-H stretching band at 2923, and that of $-CH_2$ at 1430, C-O stretching at 1080–1300, and O-H out-of-plane deformation in the 915–955 cm^{-1} range. The modified FTIR spectrum for cholesteryl-inulin (Fig. 2B) contained bands corresponding to native inulin along with new ones which were: from an isolate $-CH_3$ group at the range 1377–1383 cm^{-1}; and the characteristic frequencies of aromatic ring C-H out-of-plane deformation at 650–900 cm^{-1}. As compared to caproyl-inulin, cholesteryl-inulin had a more marked increase in its solubility in organosolvents, but no improvement was detected in its solubility in water.

The time-course analysis of batch cultures of *K. marxianus* strains IZ-275 and IZ-619, grown on native inulin and their caproyl or cholesteryl derivatives, is shown in the histograms of Figs. 3A,B (strain IZ-275 without and with phosphate supplement) and Figs. 4A,B (strain IZ-619 with and without the same supplement). The inulinase activity is expressed in terms of units (μmol of fructose$\cdot h^{-1}\cdot mL^{-1}$) of cell-free medium. Variables in the four experiments were the control (basic medium; no sugar carbon source addition), natural substrate (native inulin), or the lipophilic derivatives (caproyl- or cholesteryl-inulin) combined with the absence or presence of $NH_4H_2PO_4$ in the duplicate media.

In all instances caproyl-inulin proved to be a superior inulinase inducer, the enzyme activity in the cell-free media from a 70-h culture of strain IZ-275 being from 4.9–8.6 higher than those of the control or native inulin-based media (Figs. 3A,B). No effect was seen when the ammonium phosphate supplement was absent or present with this strain (Fig. 3A vs Fig. 3B), which gave no positive response to the cholesteryl-inulin derivative (Chol-In). IZ-619 displayed a differential behavior when submitted to

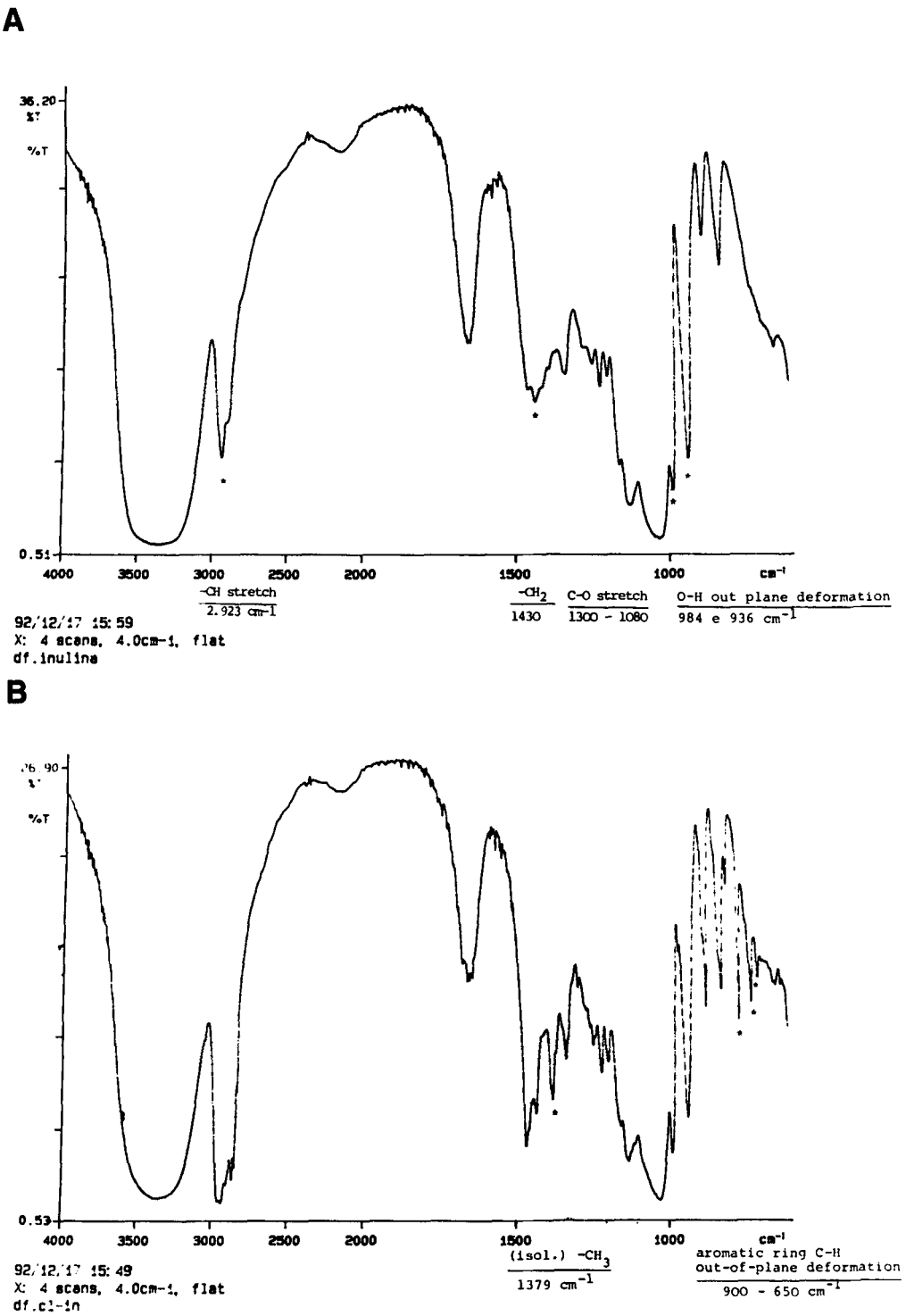

Fig. 2. (**A**) Fourier transform infrared (FTIR) spectrum of purified native inulin from *Dahlia* tubers. (Asterisk indicates typical bands of absorption for the polysaccharide.) (**B**) Fourier transform infrared (FTIR) spectrum of cholesteryl-inulin. (Asterisk indicates new absorption bands rising from the lipophilic substituent.)

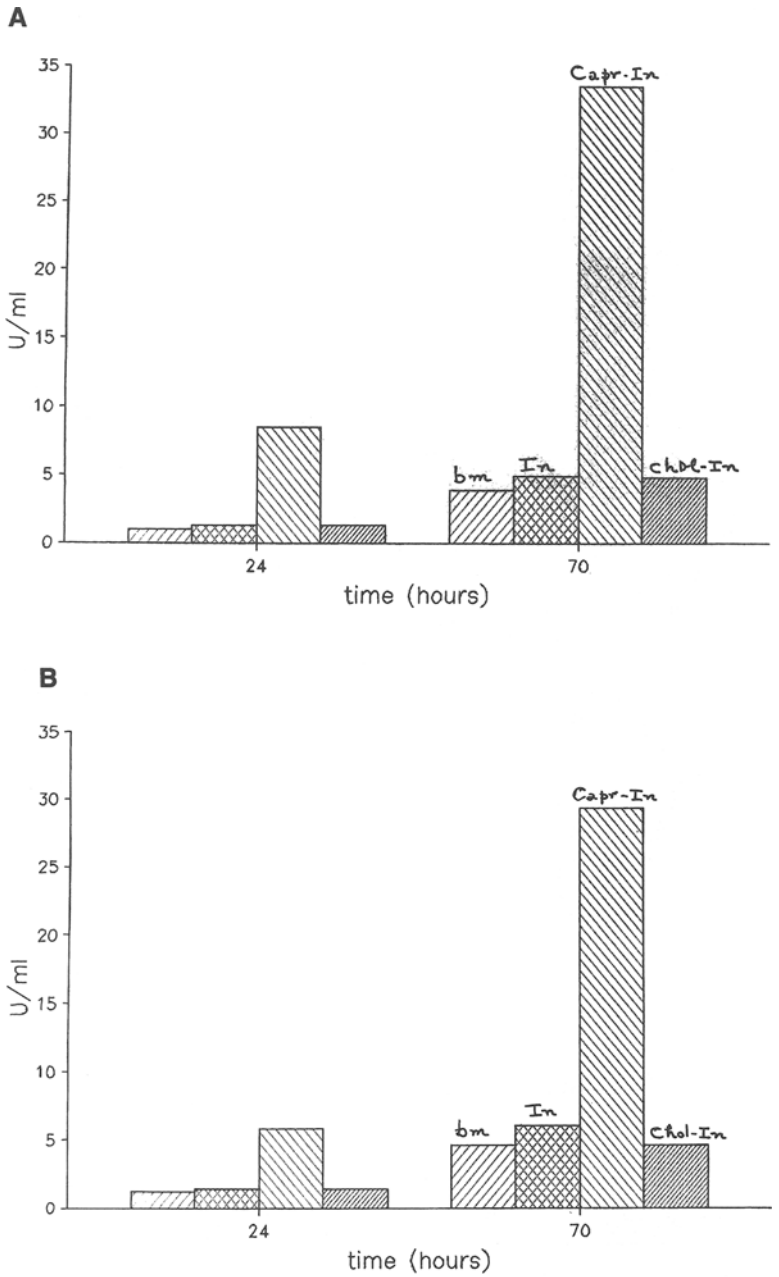

Fig. 3. The induction profile of inulinase in *Kluyveromyces marxianus* strain IZ-275. (**A**) Without ammonium dihydrogen phosphate supplement in the culture medium. (**B**) With ammonium dihydrogen phosphate supplement in the medium. (Enzyme activity expressed in units (μmol of fructose·h^{-1}·mL^{-1} of cell-free medium; column upper symbols refer to culture medium formulations without inulins [bm, basic medium], with native inulin [In], with caproyl-inulin [Capr-In] or with cholesteryl inulin [Chol-In]).

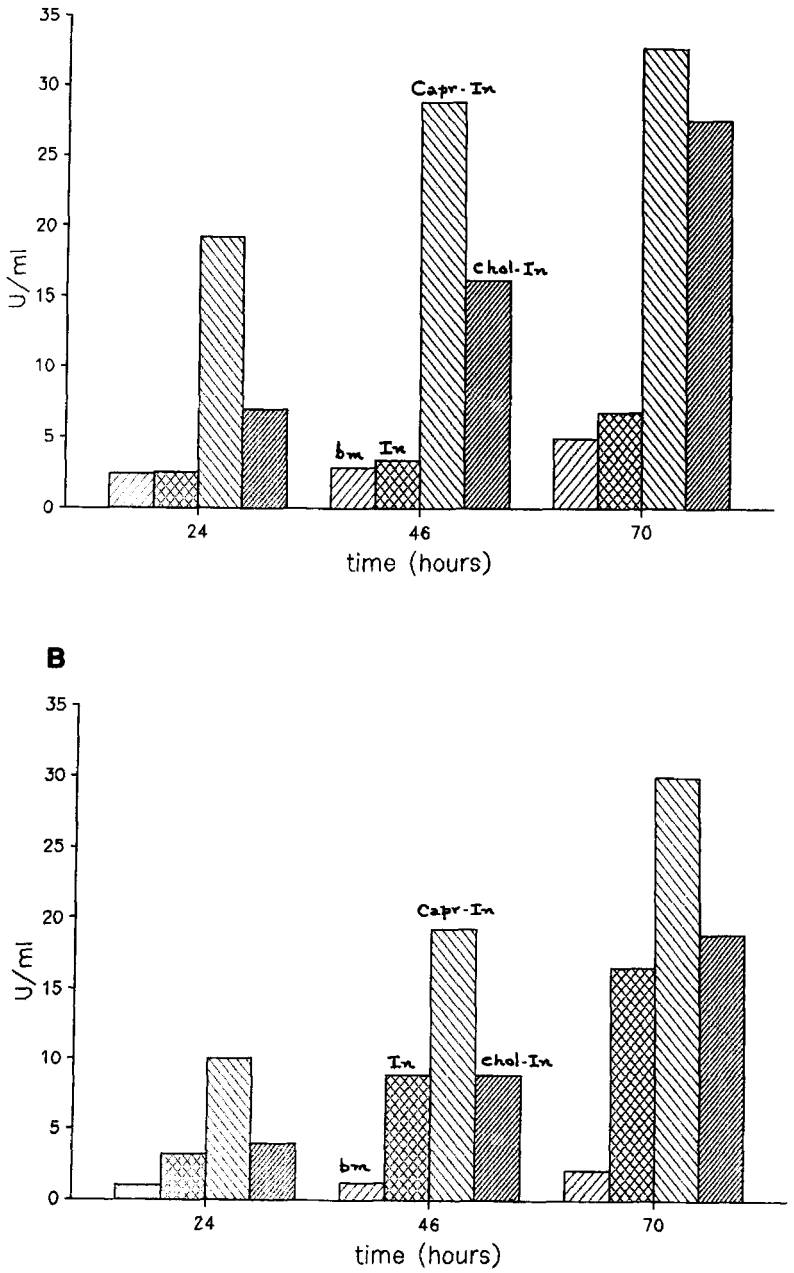

Fig. 4. The induction profile of inulinase in *Kluyveromyces marxianus* strain IZ-619. (**A**) With ammonium dihydrogen phosphate supplement in the culture medium. (**B**) Without ammonium dihydrogen phosphate supplement in the culture medium. (Other details as explained for Fig. 3.).

the same set of induction experiments. Its low basal constitutive inulinase level was more significantly increased in the presence of native inulin (3.3–7.8 times increase from 24–70 h), and ammonium phosphate presence had no beneficial effect on induction with underivatized inulin (Fig. 4A, "bm" and "In" columns), which is what indeed occurred when induction was with both derivatized inulins (Fig. 4A, columns "Capr-In" and "Chol-In", respectively 4.8- and 4.0-fold increase at 70 h as compared to native inulin induction, column "In"). Still with IZ-619 and in the absence of the salt supplement, any form of inulin proved stimulatory. Results for inulinase activity enhancement were, at 70 h, 7.8-, 13.9-, and 8.8-fold increase, respectively, for native inulin, caproyl-, and cholesteryl-derivatives (Fig. 4B), as compared to the basic inulin-free medium (Fig. 4B, column "bm"). Owing to this response level seen with native inulin, the dramatic increase of almost 14-fold with caproyl-inulin, as compared to the carbon source-deprived control, resulted in a 1.8-fold net enhancement. The best stimulatory effect of cholesteryl-inulin, both in absolute values as well as relative values to the basic media without ("bm") or with native inulin ("In"), was obtained in the presence of ammonium phosphate and only with the strain IZ-619.

It remains to be explained how the ammonium phosphate supplement so completely abolished the inductive role of native inulin on IZ strain 619. It is known, however, that the phosphate anion may play a dramatic role in the fermentation of sugars in wood hydrolyzates, in which the ratio glycerol to ethanol experienced a shift from 0.1:38.9 to 12.3:16.0 owing to the inclusion of 0.056% phosphate in the medium (*8*).

The addition of inulin as a carbohydrate source is usually a prerequisite for inulinase synthesis/secretion of microorganisms (*9*). Many are stimulated to increase their enzyme synthesis as a result of addition to the culture medium of the substrate/inducer or some structural derivative thereof. Modified or unmodified products of the enzyme action, particularly in the case of polysaccharide processing by hydrolases, may also act as enzyme inducers. The reason is because the real inducing ability of a polymeric substrate, namely the native enzyme substrate, results from its transformation into more versatile informational molecules, for instance, the disaccharides. Accordingly, if the dimer does not accumulate up to the concentration level at which repression occurs, the enzyme level will be enhanced (*10*). This fact was confirmed in studying the fungal response to cellobiose octaacetate as inducer. Its slow hydrolysis to the natural inducer, cellobiose, gave rise to a low concentration of the effective inducer over a long period of time. As a result, fungal cellulase activity increased by a factor of 4 to 100 (*11*). However, the continuous supply of limited amounts of the true inducer may be not the only or dominant reason for the improvements of enzyme level. A second factor, the surfactant effect

of the modified inducer, should be considered (*11*). Surfactants may be more easily accumulated in the cell wall membrane, thus altering its permeability, and hence facilitating the uptake of the net inducer.

Both of these considerations could explain the positive results of inulinase induction in selected yeasts using inulin previously modified by esterification with a medium-size acyl substituent (C-6; caproyl) or by etherification with the large C-27 apolar ring-fused nucleus of cholesterol. Both substituents could facilitate the anchoring of the polyfructose polymer molecule to a lipoproteic membrain domain, provided that the remaining cell wall architecture offers no steric barrier. Yeast plasma membrane crossing would be the next and decisive step for improved induction, and this could be accomplished by the whole inulin molecule or its hydrolytic fragments. Confirmation could be provided by the use of caproylated or cholesterylated low-mol-wt fructo-oligosaccharides, and work on this is currently in progress at our laboratory.

One property of caproylated-inulin, worthy of special attention, is its solubility in water, which does not occur in the native polyfructose, which needs warming but not necessarily boiling for complete solubilization. Thus, caproyl-inulin can therefore act both as a more productive inducer and also as surfactant owing to the attached aliphatic chains. How much these advantages could be further increased by variation of the present amount of bound caproyl residues, which is small considering the signals magnitude from the ^{13}C-NMR spectrum, is another question to be answered.

A broader use of derivatized inulins, especially the caproylated derivative, arose from a preliminary experiment using a prokaryotic model for comparison with the eukaryotic one of yeast. A soil bacterium feeding on rooting dahlia tubers was isolated (*3*) and it was able to produce DFA III, one of the known difructose anhydrides (dehydrated inulobioses). In vitro DFA III production from inulin could be significantly increased over a period of time in liquid culture when caproyl-inulin partially or totally replaced inulin. Cultures of 2% inulin (control), 1% inulin + 1% caproyl-inulin (half induction), and 2% caproyl-inulin (full induction), all supplemented with 0.2% yeast extract, were examined. After cell growth at 24 h at 28°C and 100 rpm, as measured as optical density at 650 nm with the washed and resuspended cells, values of 0.240, 1.900, and 2.600 U were obtained. The respective DFA III production started about 32 h in the control, but it occurred about 12 h in the induced media (DFA III content of 1.2 and 4.3 mg/mL for half- and full-induction media, respectively). No positive effect was observed in a parallel experiment using cholesteryl-inulin, but it may be recalled that prokaryotes lack sterols. Another interesting aspect to be explored with acylated inulins is the response of a filamentous mold such as *Aspergillus* whose inulinolytic apparatus differs from that of *Kluyveromyces* spp. (*13*).

We did not obtain any stimulatory effect by introducing inulin with free caproic acid as a medium combination for the studied yeasts. On the contrary, some inhibitory effect was seen in the growth and this can be attributed to the presence of free caproic acid. This was an additional indication that the positive effect of induction obtained with the inulin derivative is related to esterification of the OH groups of inulin.

CONCLUSIONS

The use of derivatized inulins proved successful regarding the increase of inulinolytic activity of yeasts. The formulation of an ideal inulinase inducer for a particular microorganism is a hard task since besides the aspects of its physiology, a large degree of substrate/inducer modifications is required. The particular aspect of substrate solubility modification arising either from the derivatization process or from any parallel small reduction of its mol wt owing to the extreme lability of the β-1,2-linked fructose polymer, will be clarified in further research.

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