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RESEARCH ARTICLE

Catalytical properties of N-glycosylated *Gluconacetobacter diazotrophicus* levansucrase produced in yeast

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Abbreviations:
FOS: fructo-oligosaccharides;
LsdA: *Gluconacetobacter diazotrophicus* levansucrase.

The influence of N-glycosylation on the kinetic and catalytical properties of a bacterial fructosyltransferase (LsdA) produced in *Pichia pastoris* was studied. The glycosylated enzyme behaved similarly to non-glycosylated LsdA when substrate specificity, fructo-oligosaccharide (FOS) production, sucrose hydrolysis or levan formation reactions were carried out under different experimental conditions. The kinetic parameters for native or yeast-expressed LsdA determined at 60ºC, condition for the highest hydrolytic activity, followed a conventional Michaelis-Menten kinetics. Synthase activity of this levansucrase increased in water-restricted environments by addition of salt or organic solvent to the reaction mixtures.

Gluconacetobacter diazotrophicus levansucrase (LsdA) catalyses four types of reactions: 1) hydrolysis, where sucrose splits into fructose and glucose, 2) Fructo-oligosaccharides (FOS) production by transferring the fructosyl residue from a donor to an acceptor sucrose molecule forming b(2 1) links, 3) polymerisation, where fructosyl residues are b(2 6) linked to a fructan polymer and 4) exchange reaction where the fructosyl residue is transferred to a glucose molecule to form sucrose, (Hernández et al. 1995).

FOS consumption benefits human and animal health by increasing bifidobacteria population in the intestinal track (Simmering and Blaut, 2001). Among them, the most attractive, 1-kestose, is produced with high yield by LsdA action on sucrose (Hernández et al. 1995; Támbara et al. 1999).

Mature native LsdA has a N-terminal pyroglutamic acid and a disulfide bridge as unique modifications (Betancourt et al. 1999) however; expression of the *lsdA* gene in *Pichia pastoris* yielded an active but glycosylated enzyme (Trujillo et al. 2001; Trujillo et al. 2002).

Structure and functional significance of glycosylation for different proteins has been increasingly appreciated (Patel et al. 1997; Montesino et al. 1998; Gemmill et al. 1999). Since LsdA constitutes a promising candidate for industrial production of FOS, we investigated the catalytic and kinetic properties of the glycosylated enzyme: i- substrate specificity, ii- product profile as a result of transfructosylations reactions on sucrose conducted in both, aqueous or water-restricted environments and iii- kinetic parameters for the hydrolysis reaction; in comparison with non-glycosylated LsdA expressed by its natural host *G. diazotrophicus* and by a recombinant *E. coli* strain.

To our knowledge, there are no reports dealing with the catalytic and kinetic properties of a recombinant glycosylated levansucrase, in aqueous or water-restricted environments.

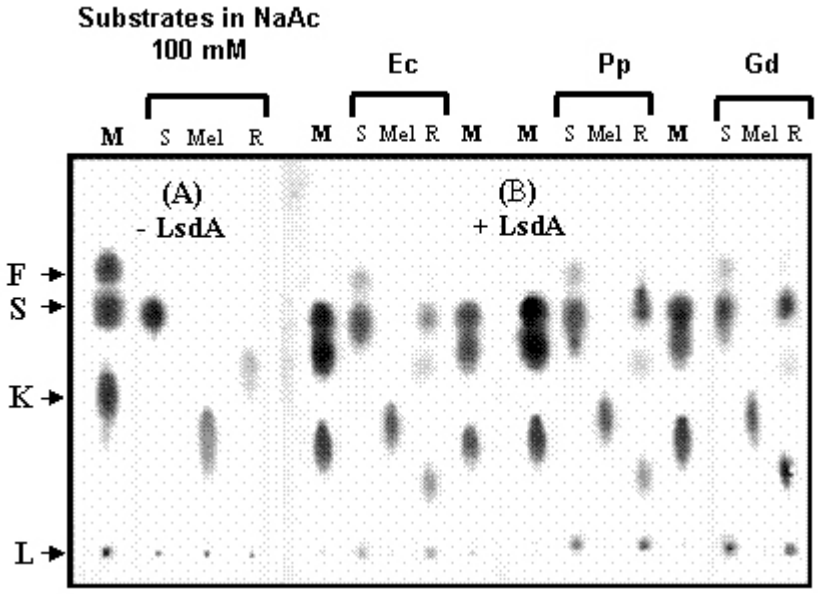

Figure 1. Substrate specificity of N-glycosylated LsdA. TLC of products of levansucrase reaction on 100 mM sucrose (S), melezitose (Mel) or raffinose (R) in buffer NaAc 100 mM, pH 5.5, incubated at 30ºC for 10 hours in the absence A) or presence B) of 5 IU of enzyme. The enzymes studied were: LsdA expressed by *P. pastoris* (Pp), natural host *Gluconacetobacter diazotrophicus* (Gd) or recombinant *E. coli* strain (Ec). Other symbols represent: (K) kestose, (L) levan and (M) sugars marker.

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Table 1. Non-linear regression statistics calculated from the hydrolysis reaction. Represented data were obtained on the basis of five independent measurements. ANOVA and signification tests (F= 1.87987, p= 0.23, significance level 0.05 and 0.01) were done using the Origin ™ Version 5 (Microcal ™) computer program.

LsdA	V_{max} (mM min^{-1})	K_m (mM)	k_{cat} (min^{-1})
G. diazotrophicus	1,73 ± 0,04	7,09 ± 0,43	59247 ± 1385
E. coli	1,67 ± 0,04	6,58 ± 0,27	57192 ± 1384
P. pastoris	1,67 ± 0,04	6,63 ± 0,34	57192 ± 1384

MATERIALS AND METHODS

Enzymes

Native levansucrase used as standard in all the biochemical and kinetics experiments was purified from culture supernatants of *G. diazotrophicus* strain SRT4 (Hernández et al. 1995).

The N-glycosylated LsdA was obtained from a recombinant *P. pastoris* as previously described (Trujillo et al. 2001; Trujillo et al. 2002). A recombinant *E. coli* strain harboring the plasmid pALS200, where the *lsdA* gene was fused at its 3' end to the myc epitope and polyhistidine tag coding sequence placed under the transcriptional control of the *npt*II promoter, was also used as a recombinant non-glycosylated source of LsdA.

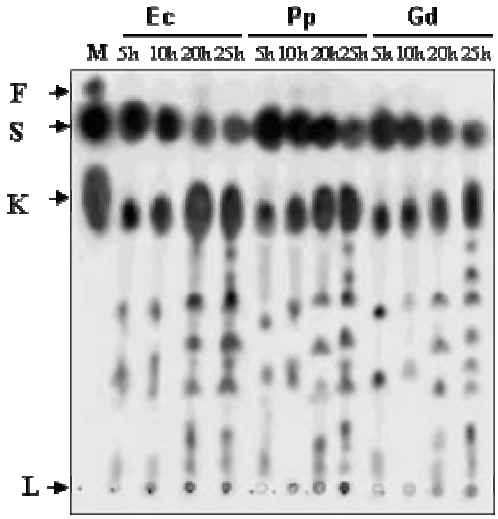

Figure 2. Time course of FOS production catalysed by glycosylated LsdA. Sucrose at 1460 mM was reacted with LsdA expressed by *P. pastoris* (Pp), natural host *G. diazotrophicus* (Gd) or recombinant *E. coli* strain (Ec), respectively, at 10 units $gram^{-1}$ of sucrose in 100 mM sodium acetate pH 5.2 at 42ºC with agitation at 200 rpm. Samples were taken at the indicated time points. Product obtained from reactions were analyzed by TLC. Symbols represent: (F) fructose, (S) sucrose, (K) kestose, (L) levan and (M) sugars marker.

Protein purification, quantification and enzyme assays

Purification of recombinant LsdA myc-His_6 produced in both, *P. pastoris* and *E. coli* was performed from culture supernatants and cell lysates respectively, by Immobilization-Metal Affinity Chromatography, using Ni-NTA agarose beads from QUIAGEN (Hilden, Germany). Total protein in culture supernatants, crude extracts and final purified preparations was determined as described by Bradford, 1976. Specific activity values of each enzymic preparation were calculated as the average of 5 different experiments. After ANOVA test, no significant differences were found between the analysed means.

LsdA purity was determined by densitometric analysis of 12.5% polyacrylamide gels stained with Coomassie brilliant blue R-250 (Sigma, USA).

Levansucrase activity was measured as the glucose released from sucrose using a glucose oxidase-peroxidase-coupled colorimetric kit (Sigma). One unit of LsdA is defined as the amount of enzyme releasing 1 µmol of glucose min^{-1} based on initial velocity measurements under the following conditions: 100 mM sucrose in 100 mM sodium acetate buffer pH 5.5, at 30ºC. The dinitrosalicylic acid (DNSA) test was also used to detect released glucose-fructose during hydrolisis reactions.

Fructan detection and quantification

Sugar samples (1 µl) were spotted on a Thin Layer Chromathography (TLC) foil (Merck KGaA, Darmstadt, Germany). TLC foils were run thrice in acetone:water (90:10) mixture and then, fructose–containing sugars were specifically stained with a urea-spray. This spray was prepared as follows: Urea (3 g) was dissolved in 100 ml of 1 M phosphoric acid in water-saturated butanol. Ethanol (5 ml) was added to eliminate the water phase which forms when the urea dissolves. TLC foils are then incubated at 100ºC for several minutes.

Total sugars in the reaction mixtures were also analysed by High Pressure Liquid Chromatography (HPLC) using an Aminex HPX-87N column (matrix DVB styrene sodium, BIO-RAD) with a refractive index detector. A solution of

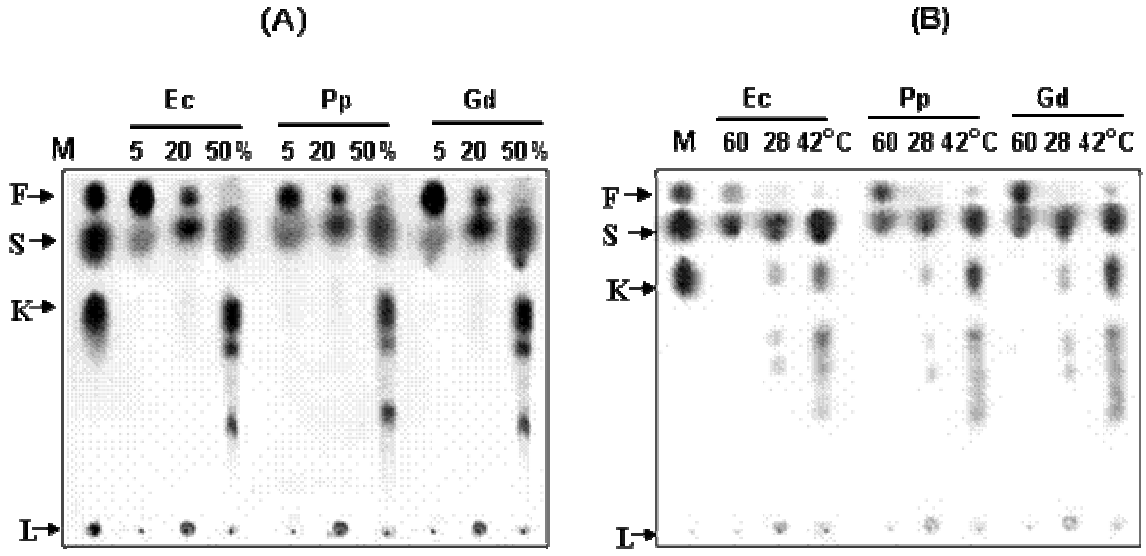

Figure 3. Influence of temperature and substrate concentration on the transfructosylation reaction of glyco-LsdA. Reactions were conducted in buffer NaAc 100 mM, pH 5.5 with 5 IU of LsdA expressed by *P. pastoris* (Pp), natural host *G. diazotrophicus* (Gd*)* or recombinant *E. coli* strain (Ec) for 10 h at 42ºC and sucrose concentrations of 146, 584 and 1460 mM A) or temperatures of 60, 28 or 42ºC and 1460 mM of sucrose B). Sugars were separated by TLC. Symbols represent: (F) fructose, (S) sucrose, (K) kestose, (L) levan and (M) sugars marker.

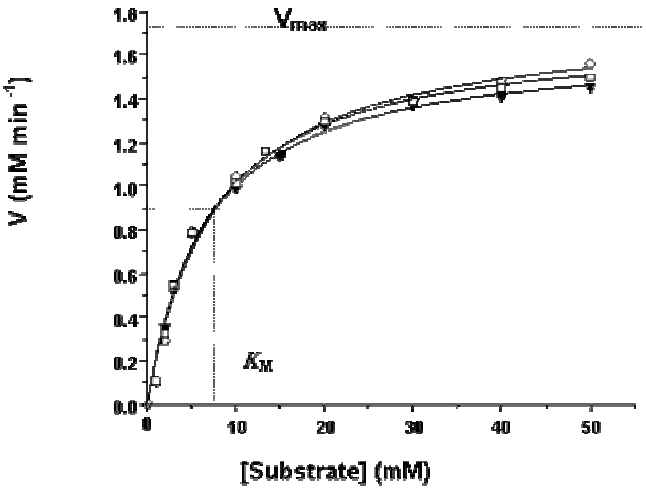

Figure 4. Initial rate kinetics of the hydrolysis reaction catalysed by LsdA. Hydrolysis reaction was carried out in a sucrose concentration range of 1 to 50 mM at 60ºC for 2 minutes in the presence of 29.2 nM of LsdA expressed by *P. pastoris* (▼), natural host *G. diazotrophicus* (O) or recombinant *E. coli* strain (□), respectively. Fructose and glucose were measured as described in methods. The initial rates were determined on the basis of five independent measures and gathered data were statistically analyzed by non-linear regression using the Origin™ Version 5 (Microcal™) computer software.

10 mM Na_2SO_4 was used as mobile phase at a flow rate of 0.5 ml min^{-1}. Known amounts of sucrose, glucose, fructose and kestose were used as standars.

RESULTS

Throughout this paper we studied the catalytical properties of glycosylated LsdA produced in *P. pastoris* however, for subsequent comparison we also included as controls in our experiments both, the native enzyme from *Gluconacetobacter diazotrophicus* (Hernández et al. 1995) and a recombinant LsdA produced in *Escherichia coli*. These two control enzymes share in common lacking N-linked carbohydrates. The three LsdA preparations displayed 95-96% purity with specific activity values of 160 ±12 U mg^{-1} calculated as described in methods.

Substrate specificity and FOS production by glycosylated LsdA

Glycosylated LsdA produced in *P. pastoris* reacted on sucrose and raffinose [O-a-D-galactopyranosyl-(1 6)-O-a-D-glucopyranosyl-(1 2)-b-D-fructofuranosidose] to form free fructose and levan in a proportion similar to those of the native or *E. coli*–produced enzymes used as controls (Figure 1). However, the enzyme did not react on melezitose [O-a-D-glucopiranosyl-(1 3)-b-D-fructofuranosyl-(2 1)-a-D-glucopiranoside], in the same fashion than native and *E. coli* expressed LsdA (Figure 1). The time course of FOS production from sucrose indicated that the glycosylated enzyme converts efficiently sucrose to FOS, mainly 1-kestose (Figure 2).

Dependence of transfructosylation reaction of glyco-LsdA on reaction conditions

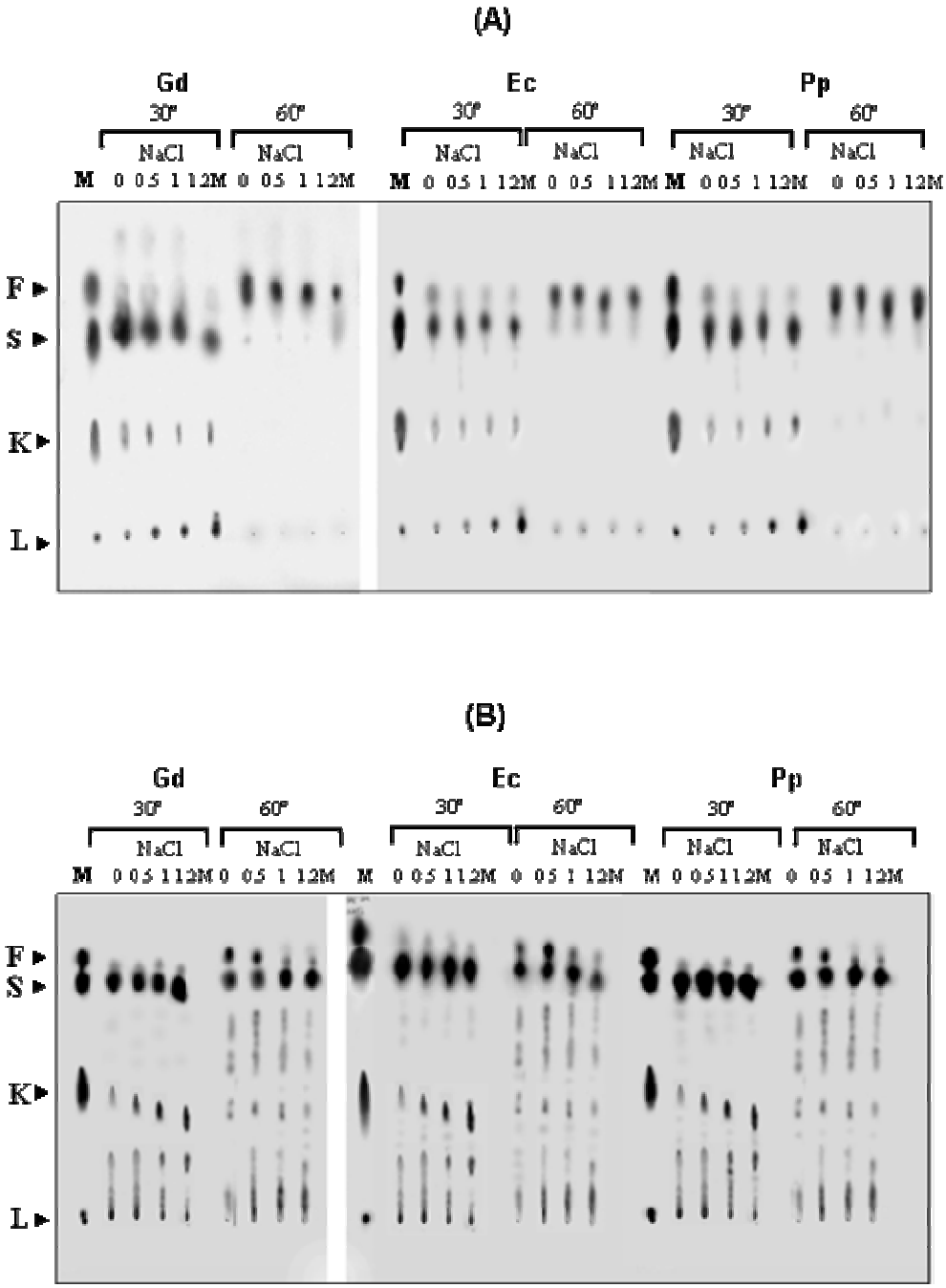

Figure 5. Effect of NaCl concentration on the transfructosylation reaction of LsdA. Sucrose at concentrations of 100 mM A) and 1500 mM B) was incubated for 10 hours in buffer NaAc 100 mM, pH 5.5, in the presence of 10 IU of LsdA expressed by *P. pastoris* (Pp), natural host *G. diazotrophicus* (Gd*)* or recombinant *E. coli* strain (Ec), at the indicated NaCl concentration and temperature. Product profiles were analyzed by TLC. Symbols represent: (F) fructose, (S) sucrose, (K) kestose, (L) levan and (M) sugars marker.

Qualitative analysis of the product profile obtained after incubation of the three enzymes under different reaction conditions showed that variations in temperature and sucrose concentration altered the product profiles of all the assayed enzymes in a similar way. (Figure 3 A, B). The highest levels of 1-kestose were produced at sucrose 50% (w/v, 1460 mM), whereas at the lowest substrate concentration (5%, 146 mM), the enzymes mainly hydrolyzed sucrose and consequently the 1-kestose levels were low (Figure 3A).

The effect of temperature on the transfructosylation reaction catalyzed by the three enzymes was examined (Figure 3B). Fructose release was maximal at 60ºC

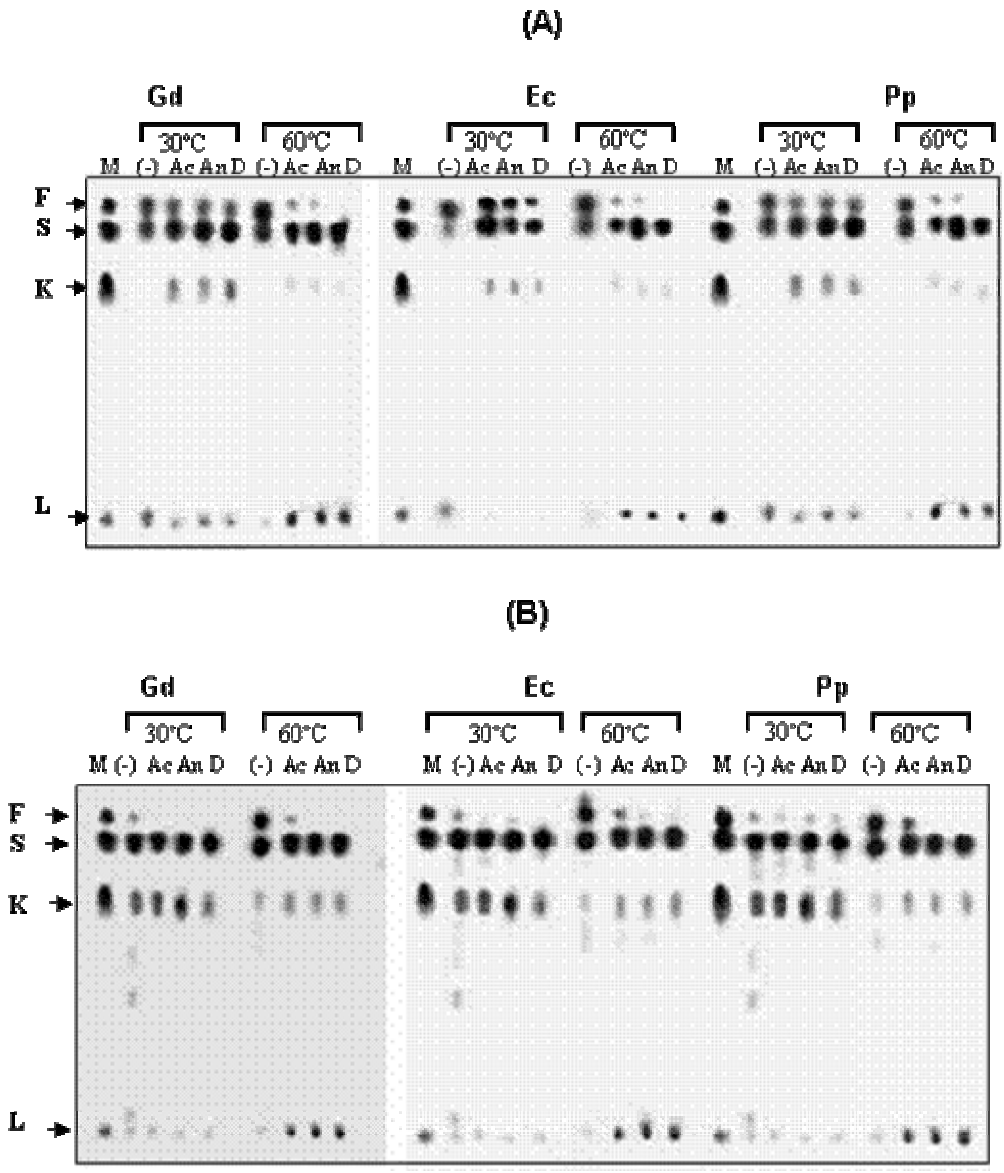

Figure 6. Effect of organic solvents on the transfructosylation reaction of LsdA. Sucrose at concentrations of 100 mM A) and 580 mM B) was incubated for 10 hours in buffer NaAc 100 mM, pH 5.5, in the presence of 10 IU of LsdA expressed by *P. pastoris* (Pp), natural host *G. diazotrophicus* (Gd*)* or recombinant *E. coli* strain (Ec) and 50% (V/V) of Acetone (Ac), Acetonitrile (An) or DMSO (D) at the indicated temperature. Product profiles were analyzed by TLC.

Symbols represent: (F) fructose, (S) sucrose, (K) kestose, (L) levan and (M) sugars marker.

indicating that the hydrolyzing activity of the assayed enzymes increased with temperature rise, while the highest conversion of sucrose to 1-kestose was reached at 42ºC. Levan formation was favoured at 28ºC. The three enzymes showed unaltered product profiles in the pH range from 5 to 8 (results not shown). The results presented in this section are in agreement to those previously reported for native *G. diazotrophicus* levansucrase (Támbara et al. 1999).

Kinetics of hydrolysis reaction catalysed by glycosylated LsdA

The influence of sucrose concentration on initial hydrolysis velocity rates (Vo) was evaluated. The chosen assay temperature of 60ºC and pH 5.5 corresponds to conditions of the highest hydrolytic activity for native and recombinant LsdA. Under these reaction conditions only released glucose and fructose were detected as reaction products after quantification of total sugars in the reaction mixtures.

Statistical analysis of data gathered from this kinetic assay showed a correctly fit with a rectangular hyperbole as

depicted in Figure 4, therefore obeying the conventional Michaelis-Menten kinetics for monosubstrate reactions where: $V_o^G = V_o^F = V_{max}$ [So] / K_m + [So]. The V_o^G and V_o^F values correspond to initial velocity of glucose and fructose released during the hydrolysis reaction, meanwhile [So] is the initial sucrose concentration. The Michaelis constant (K_m), maximal velocity (V_{max}) and catalytical constant (k_{cat}) values determined under these experimental conditions are listed in Table 1. We found no significant differences between the values of the above kinetic parameters for the three assayed enzymes as a result of its action on sucrose, indicating that N-oligosaccharides moieties of the *P. pastoris* produced enzyme affect neither catalytical velocity nor enzyme-substrate affinity. Results of kinetic studies conducted also at 30ºC did not fit a Michaelis-Menten behaviour (results not shown).

Catalytical properties of glycosylated LsdA in water–restricted environments

Water participates in the reactions catalyzed by LsdA. There are no reports dealing with catalytic properties of LsdA, neither native nor recombinant in water-restricted environments. The equilibrium between hydrolase (glucose+fructose) and synthase (levan formation) activities was determined at 60ºC and 30ºC respectively, by progressively substituting water by addition of NaCl or different organic solvents into the enzymes reaction mixture, at different substrate concentrations.

Figure 5A shows that levan formation at 30ºC increased with the increment of NaCl concentration from 0 to 1.2 M using sucrose 0.1 M as substrate. When substrate concentration was raised up to 1.5 M (Figure 5B), synthesis of 1-kestose but not polymerization was stimulated by NaCl concentration increments. Vigants and co-workers (1998) found that NaCl activated the polymerization reaction catalyzed by *Zymomonas mobilis* levansucrase in a way inversely proportional to the substrate concentration. These authors did not study FOS production under these conditions. At 60ºC, the amount of fructose liberated by LsdA decreased with increasing NaCl concentrations.

Acetone, acetonitrile and dimethyl sulfoxide share the same property of being water miscible. When sucrose concentration was 0.1 M, the presence of these organic solvents at 30ºC stimulated 1-kestose syntheses with a very low rate of levan formation, while at 60ºC, levan synthesis was enhanced. Free fructose, as a result of the hydrolase activity of the enzymes, was only detected in the absence of solvents (Figure 6A, B). When the same reactions were conducted on sucrose 0.58 M at 30ºC, no remarkable differences were observed between the products profiles obtained in aqueous or anhydrous environments, however, at 60ºC the hydrolase activity was detected only in the reaction carried out in aqueous condition, while the presence of the organic solvents stimulated the synthase activity in all the assayed enzymes.

DISCUSSION

Foreign proteins are fortuitously glycosylated when secreted in yeast. This post-traslational modification is absent in native LsdA and other levansucrases due to their bacterial origin and could affect unpredictably the enzyme activity and stability.

We studied the effect of N-glycosylation on the *Gluconacetobacter diazotrophicus* levansucrase (LsdA) recombinantly produced in *Pichia pastoris*. The predicted sequence of this recombinant enzyme contains four potential N-linked glycosylation sites, conforming to the general rule N-X-T/S where X is not proline however, the exact nature and location of the detected carbohydrate moiety have not yet been determined.

We found no effects of glycosylation on substrate specificity, FOS production, sucrose hydrolysis or levan formation by LsdA ractions carried out under different conditions. By contrast, changes in substrate specificity and protein conformation have been reported in other recombinant fructosyltransferases produced in *P. pastoris* (Hochstrasser et al. 1998; Tibbot et al. 1998).

The kinetic parameters for native or yeast-expressed LsdA determined at 60ºC, condition for the highest hydrolytic activity, followed a conventional Michaelis-Menten kinetics. The catalytic turnover rate displayed by the three assayed enzymes was approximately 10 times higher than that found for native LsdA at 30ºC (Hernández et al. 1995). The enzymes also showed higher affinity for sucrose at 60ºC (K_m= 7.1 mM) than that displayed at 30ºC (K_m=11.8 mM) previously described (Hernández et al. 1995). Interestingly, van Hijum and co-workers (2003) found that the *Lactobacillus reuteri* inulosucrase has lower affinity on sucrose during the hydrolysis reaction at 50ºC than at 20ºC.

Glycosylated LsdA does not follow a monosubstrate type of kinetics at 30ºC. These findings result quite logical since at 60ºC the enzyme acts as a hydrolase, while at 28-30ºC the enzyme catalyzes both, hydrolysis and polymerization reactions (Figure 2B) hence, at this particular temperatures the enzyme behaviour adjust better to a bisubstrate than to a monosubstrate type of kinetics.

All these results pointed out that the presence of N-linked oligosaccharides did not alter the catalytic properties of LsdA. According to 3D structural studies of levansucrases none of the four potential N-glycosylation sites are located in the active site of the ß-propeller architecture of LsdA precluding direct implications on catalysis (Meng and Futterer, 2003; Pons et al. 2004).

Although the kinetics and catalytical properties studied herein were not affected by N-glycosylation, we reported in a previous work an increment in the thermal stability of glycosylated LsdA produced in *P. pastoris* when compared with the native enzyme (Trujillo et al. 2001). The

stabilization role of carbohydrate moieties on glycoproteins has been described mainly for proteins naturally produced by fungi or yeasts (Wang and Hirs 1977; Patel et al. 1997; Boer et al. 2000). From the biotechnological standpoint is interesting the potential of most enzymes to vigorously work in organic solvents containing little or no water (Klibanov, 2003). We here show for the first time the possibility of modulating LsdA catalysis, including FOS production, by handling the concentration of organic solvents and temperature in the reaction media. *Bacillus subtilis* levansucrase is also active in water-restricted environments (Chambert and Petit-Glatron, 1989).

Industrial FOS production, to date, depends chiefly on fungal enzymes (Yun, 1996). A deeper insight into the catalytical and kinetic properties of the *P. pastoris*-produced LsdA confirms that this recombinant glycosylated enzyme represents a promising candidate for mass production of FOS from sucrose in both aqueous or water-restricted environments.

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