Substitution of Asp-309 by Asn in the Arg-Asp-Pro (RDP) motif of *Acetobacter diazotrophicus* levansucrase affects sucrose hydrolysis, but not enzyme specificity

Frank R. BATISTA, Lázaro HERNÁNDEZ¹, Julio R. FERNÁNDEZ, Juan ARRIETA, Carmen MENÉNDEZ, Raúl GÓMEZ, Yanet TÁMBARA and Tirso PONS

Centre for Genetic Engineering and Biotechnology (CIGB), P.O. Box 6162, Havana 10600, Cuba

β -Fructofuranosidases share a conserved aspartic acid-containing motif (Arg-Asp-Pro; RDP) which is absent from α -glucopyranosidases. The role of Asp-309 located in the RDP motif of levansucrase (EC 2.4.1.10) from *Acetobacter diazotrophicus* SRT4 was studied by site-directed mutagenesis. Substitution of Asp-309 by Asn did not affect enzyme secretion. The k_{cat} of the mutant levansucrase was reduced 75-fold, but its K_m was similar to that of the wild-type enzyme, indicating that Asp-309 plays a major role in catalysis. The two levansucrases showed optimal

activity at pH 5.0 and yielded similar product profiles. Thus the mutation D309N affected the efficiency of sucrose hydrolysis, but not the enzyme specificity. Since the RDP motif is present in a conserved position in fructosyltransferases, invertases, levanases, inulinases and sucrose-6-phosphate hydrolases, it is likely to have a common functional role in β -fructofuranosidases.

Key words: enzyme activity, β -fructofuranosidase, fructosyltransferase, LsdA, site-directed mutagenesis.

INTRODUCTION

The endophytic sugar-cane bacterium *Acetobacter diazotrophicus* SRT4 produces a constitutive levansucrase (LsdA; EC 2.4.1.10), which is secreted into the medium [1]. The enzyme catalyses transfructosylation from sucrose to a variety of acceptors, including water (sucrose hydrolysis), glucose (sucrose exchange), sucrose (1-kestose synthesis) and levan (polymerization). The reaction occurs via a Ping-Pong mechanism involving the formation of a transient fructosyl-enzyme intermediate. The kinetic parameters of LsdA do not differ greatly from those of the well-characterized levansucrase produced by *Bacillus subtilis*. Many other bacteria produce levansucrase; however, the catalytic properties of these enzymes have been little studied.

Comparison of the predicted primary structures of levansucrases available in the literature shows several conserved domains, which may be involved in enzyme activity. One of these domains, the Arg-Asp-Pro (RDP) motif, is also highly conserved in β -fructofuranosidases of family 32 of the glycosylhydrolases [2–4]; however, its function has not been studied to date. By means of site-directed mutagenesis, we show here that the Asp residue contained in the RDP motif of LsdA plays a significant role in catalysis.

MATERIALS AND METHODS

Materials

Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs, and radiochemicals were from Amersham. The glucose oxidase/peroxidase-coupled colorimetric kit (Trinder) was from Sigma.

Bacterial strains and media

Escherichia coli strains used were: MC 1061 [F^- *araD139* Δ (*ara-leu*)7696 Δ (*lac*)X74 *galU galK hsdR2* ($r_K^* m_K^*$) *mcrBI rpsL*

(Str^r)] [5], NM522 [*supE thi-1 (hsdMS-mcrB)5* Δ (*lac-proAB*) [*mutS::Tn10*] *F'*[*proAB⁺ lacI^r* Δ (*lacZ*) *M15*] ($r_K^* m_K^* mcrB^-$)] [6], and S17-1 [*thi pro hsdR recA* [RP4-2(Tc::Mu) (*Km::Tn7*)] [7]. *Acetobacter diazotrophicus* strains used were: SRT4 (wild-type, Lev⁺) [8] and AD1 (SRT4 *lsdA::nptII-ble* cassette, Lev⁻) [9]. *E. coli* strains were grown in Luria broth supplemented with ampicillin (50 μ g/ml) and tetracycline (12.5 μ g/ml) as appropriate. *A. diazotrophicus* strains were grown in LGIE medium [9]. Chloramphenicol (30 μ g/ml) and tetracycline (20 μ g/ml) were added to LGIE agar medium to select the transconjugants.

Site-directed mutagenesis

Plasmid pALS5 [9] containing the *lsdA* gene under its own promoter was used as a template for mutagenesis using the Unique-Site Elimination Mutagenesis Kit (Pharmacia-Biotech). The oligonucleotide 5'-TTCTTCAATTCGCCAATCCGTTCA-CCTTC-3' was used to generate the mutation D309N in the *lsdA* gene. The mutant strand was selected using the mutation introduced by the oligonucleotide 5'-CGCGCGCATCAGGT-CGGATATCGTTTCGTTTCAGGTAGG-3', which changed the unique *NcoI* site in pALS5 into an *EcoRV* unique site (mismatches are underlined). After complementary strand synthesis and ligation, the DNA was digested with *NcoI* and used to transform *E. coli* NM522 (*mutS*) cells. Plasmids from transformants were mass-purified, digested with *NcoI* and used to transform *E. coli* MC 1061. Mutants were screened by *EcoRV* digestion, and the D309N substitution was confirmed by DNA sequencing. The plasmid carrying the mutated *lsdA* gene was named pALS92.

Expression of recombinant LsdA in a levansucrase-deficient *A. diazotrophicus* strain

Plasmids pALS5 and pALS92 were *HindIII*-linearized and inserted in the broad-host-range cloning vector pRK293 [10]. The

Abbreviation used: LsdA, levansucrase from *Acetobacter diazotrophicus*.

¹ To whom correspondence should be addressed (e-mail ipmlab@cigb.edu.cu).

Table 1 Conserved motifs containing acidic residues in bacterial fructosyltransferases

Conserved acidic residues are in **bold**. At the bottom, asterisks indicate identity and dots indicate conservative changes (STA; LVIM; KR; DE; QN; FYW). Numbering refers to the precursor form of *A. diazotrophicus* LsdA.

Source	Accession no.	Motifs				
		I	II	III	IV	V
<i>Acetobacter diazotrophicus</i>	L41732	V ¹³³ WDTW	E ²²³ WSG	F ³⁰⁷ RDP	V ³²⁵ FEG	V ³⁹⁶ NDQTER
<i>Zymomonas mobilis</i>	L08093	VWDTW	EWSG	FRDP	LFEG	VNDQTER
<i>Erwinia amylovora</i>	X75079	IWDTM	EWAG	FRDP	LFEG	VNDQTER
<i>Streptococcus salivarius</i>	L08445	VWDSW	QWSG	LRDP	IFES	VTDEVER
<i>Streptococcus mutans</i>	M18954	VWDSW	EWSG	MRDP	VFEA	VSDELER
<i>Bacillus subtilis</i>	A25040	VWDSW	EWSG	LRDP	VFEA	VTDEIER
<i>Bacillus amyloliquefaciens</i>	P21130	VWDSW	EWSG	LRDP	VFEA	VTDEIER
<i>Bacillus stearothermophilus</i>	P94468	VWDSW	EWSG	LRDP	VFEA	VTDEIER
		●●●●	★●★	●★★★	●★★	★★★

Table 2 Catalytic behaviour of D309N LsdA in comparison with the native enzyme

The reaction mixtures (50 μ l) containing 200 mM sucrose in 0.1 M sodium acetate, pH 5.0, were incubated with 0.3 unit of purified enzyme at 30 °C. After 15 min, the reaction was stopped by heating in a boiling-water bath for 5 min. Glucose released from sucrose hydrolysis was measured by a glucose oxidase/peroxidase-coupled colorimetric reaction. The catalytic constant (k_{cat}) was calculated using 60 000 as the M_r of a functional unit of levansucrase. The K_m value for sucrose was determined within a substrate concentration range of 1–150 mM at 30 °C and pH 5.0. The kinetic parameters were determined by non-linear regression analysis of substrate–velocity plots using the LEONORA program [18]. One unit of enzyme activity is defined as the amount of enzyme releasing 1 μ mol of glucose/min based on initial-velocity measurements under the following conditions: 0.2 M sucrose in 0.1 M sodium acetate buffer, pH 5.0, at 30 °C. Shown are the mean values from ten independent experiments.

LsdA	k_{cat} (min ⁻¹)	K_m (mM)	k_{cat}/K_m (M ⁻¹ ·min ⁻¹)
Wild-type	$(3.6 \pm 0.5) \times 10^3$	11.4 ± 0.7	3.2×10^5
D309N	47.8 ± 1.2	10.5 ± 0.9	4.5×10^3

resulting plasmids were named pALS6 and pALS93 respectively, and were used to transform *E. coli* S17-1 (*tra*⁺). The conjugal mating procedure as described by Arrieta et al. [9] was used to mobilize plasmids pALS6 and pALS93 into the *A. diazotrophicus* mutant AD1, in which the *lsdA* gene is disrupted by the *nptII-ble* cassette.

Sequence analysis

The database searches in EMBL, SWISS-PROT and PIR were performed using Blast [11] and profile search programs [12], both using the default parameters. The ClustalW program [13] was used to create a consensus multiple alignment of glycosylhydrolases (family 32) and bacterial fructosyltransferases.

Other methods

The mutated *lsdA* gene in pALS92 was sequenced using the dideoxy chain-termination method [14] with a Sequenase 2.0 sequencing kit (Amersham). The native and mutant levansucrases were purified from culture supernatants as described by Arrieta et al. [9]. Purified proteins were quantified by the Bradford procedure [15] using BSA as a standard. Proteins in culture supernatants were quantified by densitometric analysis of 12.5 % (w/v) polyacrylamide gels stained with Coomassie Brilliant Blue

Figure 1 Expression and purification of D309N LsdA analysed by SDS/PAGE

Proteins were analysed on an SDS/12% (w/v)-polyacrylamide gel. Samples were denatured by heating at 100 °C for 5 min in 2% (w/v) SDS and 143 mM β -mercaptoethanol before electrophoresis. Proteins were revealed by Coomassie Blue staining. Lanes: 1, protein molecular mass markers (kDa); 2, culture supernatant from the *lsdA::nptII-ble A. diazotrophicus* mutant AD1 harbouring pALS93; 3, purified D309N LsdA.

R-250 (Sigma). SDS/PAGE was performed as described by Laemmli [16]. For Western blotting, proteins were separated by SDS/PAGE, transferred on to a nitrocellulose membrane and probed with rabbit polyclonal antibodies against native LsdA [9], and binding was detected using an anti-[rabbit IgG (Fc)]–alkaline phosphatase conjugate (Promega).

RESULTS AND DISCUSSION

Acidic residues have been found to be responsible for catalysis in most glycosylhydrolases studied to date [17]. Multiple sequence alignment of bacterial fructosyltransferases available in the EMBL/SWISS-PROT and PIR databases revealed the presence of five well-conserved domains containing aspartate or glutamate residues (Table 1). Substitution of Asp-309 by Asn in domain III (the RDP motif) of LsdA was carried out by site-directed mutagenesis. A single base change (AAT instead of GAT in the codon corresponding to Asp-309) was generated in the mutated *lsdA* gene, as confirmed by DNA sequencing. Levansucrase expression from plasmid pALS6 or pALS93 in the *lsdA::nptII-ble A. diazotrophicus* mutant AD1 resulted in a secreted protein with the expected molecular mass of 60 kDa, as deduced by SDS/PAGE and Western blot analysis. Densitometric analysis of the supernatant proteins separated on SDS/polyacrylamide gels revealed that the native and mutant levansucrases both represented over 80 % of the total secreted proteins. This finding

Table 3 Sequence comparison of several β -fructofuranosidases containing the conserved RDP motif

The RDP motif is shown in **bold**. Abbreviations: FTF, fructosyltransferase; sucrose-6-P, sucrose 6-phosphate.

Enzyme	Source	RDP motif	Accession no.
Levansucrase	<i>Acetobacter diazotrophicus</i>	N FRDP FTFE	L41732
Levansucrase	<i>Zymomonas mobilis</i>	D FRDP HVFI	L08093
Levansucrase	<i>Erwinia amylovora</i>	N FRDP SPFI	X75079
Levansucrase	<i>Streptococcus mutans</i>	A MRDP HVIE	M18954
Levansucrase	<i>Bacillus subtilis</i>	T LRDP HYVE	A25040
Levansucrase	<i>Bacillus amyloliquefaciens</i>	T LRDP HYVE	P21130
Levansucrase	<i>Bacillus stearothermophilus</i>	T LRDP HYVE	P94468
Fructosyltransferase	<i>Streptococcus salivarius</i>	C LRDP HIIE	L08445
Sucrose:fructan 6-FTF	<i>Hordeum vulgare</i>	D FRDP MTAW	Q96466
Fructan:fructan 6G-FTF	<i>Allium cepa</i>	D FRDP NPIW	P92916
Levanase	<i>Bacteroides fragilis</i>	D FRDP KVFW	M83774
Levanase	<i>Bacillus subtilis</i>	D FRDP KVFW	P05656
Levanase	<i>Bacillus polymyxa</i>	D FRDP KVIW	A55206
Levanase	<i>Bacillus stearothermophilus</i>	D FRDP KVIW	U34875
Endo-inulinase	<i>Penicillium purporogenum</i>	E S RD PKVFF	D84360
Inulinase	<i>Kluyveromyces marxianus</i>	N FRDP KVFW	P28999
Raffinose invertase	<i>Escherichia coli</i>	H FRDP KV-W	P16553
Invertase	<i>Saccharomyces cerevisiae</i>	Q FRDP KVFW	P10594
Invertase	<i>Hansenula anomala</i>	Q QRDP KVLW	P40912
Invertase	<i>Phaseolus aureus</i>	D FRDP TTAW	P29001
Invertase	<i>Solanum tuberosum</i>	D FRDP TTAW	L29099
Invertase	<i>Daucus carota</i>	D FRDP TTAW	X67163
Invertase	<i>Vicia faba</i>	D FRDP TTAW	Z49831
Invertase	<i>Zea mays</i>	D FRDP TTVL	P49175
Invertase	<i>Arabidopsis thaliana</i>	S FRDP TTAW	X74514
Sucrose-6-P hydrolase	<i>Vibrio alginoliticus</i>	H FRDP KV-W	M26511
Sucrose-6-P hydrolase	<i>Zymomonas mobilis</i>	H FRDP RV-W	M62718
Sucrose-6-P hydrolase	<i>Klebsiella pneumoniae</i>	H VRDP KV-W	P27217
Sucrose-6-P hydrolase	<i>Escherichia coli</i>	H FRDP KV-W	P40714
Sucrose-6-P hydrolase	<i>Streptococcus xilosus</i>	H FRDP KV-H	Q05936
Sucrose-6-P dehydrogenase	<i>Vibrio cholerae</i>	H IRDP KV-I	M74035

Figure 2 Effect of pH on enzymic activity of D309N LsdA

The effect of pH was examined using 0.1 M sodium acetate buffer in the pH range 3.0–6.0 (▲) and sodium phosphate buffer in the pH range 6.0–8.0 (■) at 30 °C. Enzyme activity was evaluated using the sucrose hydrolysis reaction described in Table 2. Each enzyme activity assay was replicated three times.

indicates that substitution of Asp-309 by Asn did not affect enzyme synthesis or secretion.

The native and mutant proteins were purified to homogeneity from *A. diazotrophicus* supernatants (Figure 1) and their kinetic properties were determined. As shown in Table 2, the k_{cat} value for the D309N LsdA was decreased 75-fold, but its K_{m} value was

Figure 3 HPLC chromatograms of the reaction products obtained from sucrose with native (A) and mutant (B) LsdA

The reaction mixtures (15 ml) containing 1 M sucrose (initial concentration) in 0.1 M sodium acetate, pH 5.0, were incubated with 25 units of enzyme (see the legend to Table 2) at 30 °C with shaking at 200 rev./min. After 6 h, the reaction was stopped by heating in a boiling-water bath for 5 min. Aliquots of each reaction mixture were diluted appropriately and analysed by HPLC using an Aminex HPX-87N column [0.78 cm (internal diam.) × 30 cm; Bio-Rad] with a refractive index detector. A solution of 10 mM Na₂SO₄ was used as the mobile phase at a flow rate of 0.5 ml/min. The operating temperature was 85 °C. F, G, S, K and DP > 3 refer respectively to fructose, glucose, sucrose, 1-kestose and fructans with degree of polymerization greater than 3.

very similar to that of the wild-type enzyme, indicating that Asp-309 plays an essential role in the catalytic mechanism of LsdA. The activity of the mutant enzyme was optimal at pH 5.0 (Figure 2), as reported previously for native LsdA [1]. The transfructosylation reaction of the two levansucrases rendered similar product profiles, with the accumulation of 1-kestose being relevant (Figure 3). These results indicate that the mutation D309N in LsdA, although it affected the efficiency of sucrose hydrolysis, did not alter enzyme specificity.

The RDP motif is found at a conserved position, not only in levansucrases but also in invertases, levanases, inulinases and sucrose-6-phosphate hydrolases (Table 3); however, it is absent from α -glucosidases and α -glucosyltransferases. Two acidic residues have been identified as being involved in the catalytic mechanism of yeast invertase: Asp-23 as a nucleophile and Glu-204 as an acid/base catalyst [19,20], but the function of the RDP motif has not been studied to date. It is known that, within a family or related families, highly conserved amino acids are usually associated with either a structural or a functional role. On this basis, the catalytic importance of the RDP motif in LsdA may extend to other β -fructofuranosidases.

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Received 21 September 1998; accepted 28 October 1998