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Expression of Bifunctional Enzymes with Xylose Reductase and Xylitol Dehydrogenase Activity in *Saccharomyces cerevisiae* Alters Product Formation during Xylose Fermentation

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To enhance metabolite transfer in the two initial sequential steps of xylose metabolism in yeast, two structural genes of *Pichia stipitis*, *XYL 1* and *XYL 2* encoding xylose reductase (XR) and xylitol dehydrogenase (XDH), respectively, were fused in frame. Four chimeric genes were constructed, encoding fusion proteins with different orders of the enzymes and different linker lengths. These genes were expressed in *Saccharomyces cerevisiae*. The fusion proteins exhibited both XR and XDH activity when *XYL 1* was fused downstream of *XYL 2*. The specific activity of the XDH part of the complexes increased when longer peptide linkers were used. Bifunctional enzyme complexes, analyzed by gel filtration, were found to be tetramers, hexamers, and octamers. No degradation products were detected by Western blot analysis. *S. cerevisiae* strains harboring the bifunctional enzymes grew on minimal-medium xylose plates, and oxygen-limited xylose fermentation resulted in xylose consumption and ethanol formation. When a fusion protein, containing a linker of three amino acids, was coexpressed with native XR and XDH monomers in *S. cerevisiae*, enzyme complexes consisting of chimerical and native subunits were formed. The total activity of these complexes showed XR and XDH activities similar to the activities obtained when the monomers were expressed individually. Strains which coexpressed chimerical subunits together with native XR and XDH monomers consumed less xylose and produced less xylitol. However, the xylitol yield was lower in these strains than in strains expressing only native XR and XDH monomers, 0.55 and 0.62, respectively, and the ethanol yield was higher. The reduced xylitol yield was accompanied by reduced glycerol and acetate formation suggesting enhanced utilization of NADH in the XR reaction. © 2001 Academic Press

INTRODUCTION

In xylose-utilizing yeasts the enzymes xylose reductase, EC 1.1.1.21 (XR), and xylitol dehydrogenase, EC 1.1.1.14 (XDH), convert xylose to xylulose via xylitol (Rizzi *et al.*, 1988, 1989). In recombinant *Saccharomyces cerevisiae*-expressing XR and XDH, xylose fermentation is characterized by slow and incomplete sugar consumption and high xylitol secretion (Kötter *et al.*, 1993; Tantirungkij *et al.*, 1993; Walfridsson *et al.*, 1995). This has been interpreted as a result of XR preferentially using NADPH as cofactor, whereas XDH exclusively uses NAD^+, so that xylitol excretion occurs as a result of NAD^+ depletion (Bruinenberg *et al.*, 1983, 1984). Consistent with this interpretation is the observation that xylitol production and secretion in recombinant *S. cerevisiae* cells increased in the absence of oxygen as an electron acceptor for NADH (Tantirungkij *et al.*, 1993, 1994; Walfridsson *et al.*, 1995).

The K_m of *Pichia stipitis* XR is 10 times higher for NADH than for NADPH (Rizzi *et al.*, 1988). However, heterologous XR expressed in *S. cerevisiae* is able to use NADH as cofactor (Lidén *et al.*, 1996). The production of glycerol, which is due to reoxidation of surplus NADH originating from biomass production (Albers *et al.*, 1996; Oura, 1977), was reduced in strains expressing *XYL1* only, indicating NADH consumption (Meinander *et al.*, 1996). Additionally, a mutant of *S. cerevisiae* expressing *XYL1* and incapable of glycerol production due to deletion of genes *GPD1* and *GPD2* encoding glycerol-3-phosphate dehydrogenase was capable of anaerobic glucose conversion in the presence of xylose (Lidén *et al.*, 1996).

Xylitol secretion might be reduced and the xylitol flux through the pentose phosphate pathway increased by introducing an artificial bifunctional enzyme with XR and XDH activity. Theoretical analyses have shown that such a bifunctional enzyme may lead to channeling or proximity effects with a reduction of the intermediates' transient

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time as a result (Easterby, 1981; Keleti and Welch, 1984; Kholodenko *et al*., 1996a, b). The proximity between two sequentially operating enzymes has been considered advantageous because it may circumvent unfavorable equilibrium by creating higher local concentrations of the intermediates around the enzyme complex (Ovadi, 1991; Srere, 1987). In genetically prepared bifunctional enzymes, kinetic advantages have been demonstrated in pairs of sequentially operating enzymes (Bülow, 1987; Carlsson *et al*., 1996; De Moraes *et al*., 1995; Lindbladh *et al*., 1992; Ljungcrantz *et al*., 1989; Shibuya *et al*., 1992).

Bifunctional enzymes with XR and XDH activities might reduce xylitol formation and increase ethanol formation by creating xylitol channeling and high local concentrations of NADH around the enzyme. NADH/NAD^+ would be continuously recycled between the site of oxidation (XR) and the site of reduction (XDH). Oxidation of NADH instead of NADPH would be stimulated and xylitol secretion would be prevented. In this paper, we report the construction of strains of *S. cerevisiae* expressing artificial genes which encode bifunctional enzymes, with XR and XDH activity, as well as strains that produce bifunctional enzymes together with native monomers of XR and XDH. We have determined the specific activities, the influence of the length of the linker, and the aggregation properties of the enzyme complexes. We have also compared the different strains with respect to their ability to ferment xylose.

MATERIALS AND METHODS

Strains and Media

Escherichia coli DH5α[F^-*Φ*80d*lacZ* DM15 D(*lacZYA-argF*) U169 *deoR recA1 endA1 hsdR17*($r_k^-m_k^+$) *supE44* λ^- *thi-1 gyrA96 relA1*] (GIBCO BRL, Gaithersburg, MD) was used for subcloning. *S. cerevisiae* GPY55-15Bα(*leu2-3 leu2-112 ura3-52 trp1-289 his4-519 prb1 cir*$^+$) (Gregg Payne, University of California, Berkeley, CA), was used as a host for transformation. The recombinant yeast strains used in this study are listed in Table 1. Yeast strains were grown in a synthetic complete (SC) medium (Sherman *et al*., 1983) (0.067% Difco yeast nitrogen base without amino acids, supplemented with 13.5 mg/L adenine, 348 mg/L arginine, 266 mg/L aspartic acid, 58 mg/L histidine, 36 mg/L inositol, 525 mg/L isoleucine, 262 mg/L leucine, 91 mg/L lysine, 149 mg/L methionine, 83 mg/L phenylalanine, 105 mg/L serine, 119 mg/L threonine, 82 mg/L tryptophan, 18 mg/L tyrosine, 22 mg/L uracil, and 117 mg/L valine). For selection of the transformants, either uracil or leucine or uracil and leucine were excluded from the medium. *E. coli* transformants were grown in L-broth (Sambrook *et al*., 1989) containing 100 mg/ml ampicillin.

DNA Manipulation and Transformation

Plasmid manipulation, plasmid DNA isolation, agarose gel electrophoresis, purification of DNA fragments, and transformation were performed according to standard protocols (Sambrook *et al*., 1989). PCR was performed as recommended by the manufacturer, and the sequences of the PCR-amplified fragments were verified by DNA sequencing. Restriction enzymes, *Pwo* DNA polymerase, and T4 DNA ligase were purchased from Roche Molecular Biochemicals (Roche Diagnostics Scandinavia AB, Bromma, Sweden) and used as recommended by the manufacturer. Yeast cells were made competent for plasmid uptake by treatment with lithium acetate and polyethylene glycol (Schiestl and Gietz, 1989).

Construction of Plasmids

Plasmids used for cloning and for construction of the chimerical genes were pMW103 and pMW104 (Walfridsson *et al*., 1995), YEp24-PGK (Walfridsson *et al*., 1997), and pUC19 (Yanisch-Perron *et al*., 1985). Plasmids pMW103 and pMW104 are based on the yeast expression vector pMA91 (Mellor *et al*., 1983) containing the *XYL1* and *XYL2* genes from *P. stipitis* under the control of the promoter and transcription termination regions of the yeast phosphoglycerate kinase (*PGK1*) (Mellor *et al*., 1983) and alcohol dehydrogenase 1 (*ADH1*) genes (Ammerer, 1983). YEp24-PGK was the expression vector YEp24 (Rose *et al*., 1990) where two of three *Hin*dIII sites have been deleted. This plasmid contains a 1.86-kb *Hin*dIII fragment with the promoter and transcription termination regions of the yeast *PGK1* gene (Mellor *et al*., 1983) separated by a *Bgl*II site. Nine primers for PCR amplification of the *XYL1* and *XYL2* genes were designed and used for the construction of the chimerical genes. The sequences are

Primer 1: 5′-CGCAGGATCCACTAGAATGCCTTCTAT-3′;

Primer 2: 5′-TCCTCTAGATTGGACGAAGATAGGAAT-3′;

Primer 3: 5′-GCGTCTAGAATGACTGCTAACCCTTCC-3′;

Primer 4: 5′-GCGCGAAGCTTAGATCTT TACTCAG GGCCGTCAA-3′;

Primer 5: 5′-GCCTCTAGAATGCCTTCTATTAAG-3′;

Primer 6: 5′-GCGCGAAGCTTGGATCCTTAGACGA AGATAGGAA-3′;

Primer 7: 5′-CGCAGGATCCACTAGAATGACTGCT AACCCTTC-3′;

TABLE 1

Recombinant Strains of *S. cerevisiae* GPY55-15Bα Containing Plasmids Indicated

Strain	Plasmid(s)	Expressed gene(s)[a]	Source or reference
G-104	pMW104	*XYL1* and *XYL2*	Walfridsson *et al.* (1995)
G-YEpPGK	YEp24-PGK	—	Walfridsson *et al.* (1997)
G-104/YEpPGK	pMW104 and YEp24-PGK	*XYL1* and *XYL2*	This work
G-XRXDH-S	pXRXDH-S	*XYL1–XYL2* (short linker)[b]	This work
G-XDHXR-S	pXDHXR-S	*XYL2–XYL1* (short linker)[c]	This work
G-XDHXR-M	pXDHXR-M	*XYL2–XYL1* (medium linker)[d]	This work
G-XDHXR-L	pXDHXR-L	*XYL2–XYL1* (long linker)[e]	This work
G-104/XDHXR-S	pMW104 and pXDHXR-S	*XYL1* and *XYL2*, *XYL2–XYL1* (short linker)[c]	This work

[a] Abbreviations used: *XYL1*, gene encoding xylose reductase (XR); *XYL2*, gene encoding xylitol dehydrogenase (XDH).
[b] Gene encoding chimeric fusion protein with linker encoding three amino acids (Gln-Ser-Arg) and XR at the N-terminal and XDH at the C-terminal end of the protein.
[c] Gene encoding chimeric fusion protein with linker encoding three amino acids (Gly-Ser-Arg) and XDH at the N-terminal and XR at the C-terminal end of the protein.
[d] Gene encoding chimeric fusion protein with linker encoding six amino acids (Gly-Ser-Arg-Thr-Asn-Gln) and XDH at the N-terminal and XR at the C-terminal end of the protein.
[e] Gene encoding chimeric fusion protein with linker encoding 12 amino acids (Gly-Ser-Arg-Pro-Ser-Pro-Thr-Ala-Ser-Thr-Asn-Gln) and XDH at the N-terminal and XR at the C-terminal end of the protein.

Primer 8: 5′-TCCTCTAGAACCCTCAGGGCCGTCAATG-3′; and

Primer 9: 5′-GCCTCTAGACCATCTCCAACCGCTAGCACTAACCAAATGCCTTCTATTAAGTTG-3′.

Primer 1, which contains a *Bam*HI site in the 5′-terminal end, corresponds to the 5′-end of the *XYL1* gene. Primer 2 contains an *Xba*I site and hybridized to the 3′-terminal end of the *XYL1* gene so that the translational stop codon was removed in the amplified gene. *XYL1* was amplified by PCR from plasmid pMW103, digested with *Bam*HI and *Xba*I, and inserted into a similarly cleaved pUC19. Primer 3 contains an *Xba*I site and primer 4 contains both *Bgl*II and *Hin*dIII sites and hybridized to the 5′-terminal and the 3′-terminal ends of the *XYL2* gene, respectively. *XYL2* was amplified from plasmid pMW103, digested with *Xba*I and *Hin*dIII, and inserted downstream of the *XYL1* gene in the pUC19 previously digested with *Xba*I and *Hin*dIII.

To obtain an expression vector for use in *S. cerevisiae*, strain GPY55-15Bα, pUC19 containing the gene encoding the fusion protein was digested with *Bam*HI and *Bgl*II and moved into the *Bgl*II-digested expression vector YEp24-PGK. The resulting plasmid pXRXDH-S encoded the entire XR, a short linker of three amino acids (Gln-Ser-Arg), followed by the entire XDH. A similar strategy was followed to design plasmids pXDHXR-S and pXDHXR-L (Table 1).

Plasmid pXDHXR-S was constructed using primers 5 and 6 corresponding to the 5′- and 3′-ends of the *XYL1* gene, respectively, and primers 7 and 8 corresponding to the 5′-and 3′-ends of the *XYL2* gene, respectively. The resulting plasmid encoded the entire XDH, a short linker of three amino acids (Gly-Ser-Arg), followed by the entire XR.

Plasmid pXDHXR-L was constructed using primers 5 and 6 and primers 7 and 9, corresponding to *XYL1* and *XYL2*, respectively. pXDHXR-L encoded the entire XDH, a long linker of 12 amino acids (Gly-Ser-Arg-Pro-Ser-Pro-Thr-Ala-Ser-Thr-Asn-Gln), followed by the entire XR.

Finally, plasmid pXDHXR-M codes for an in-frame fusion of *XYL2* and *XYL1*, with six amino acids (Gly-Ser-Arg-Thr-Asn-Gln) in the connecting region. This was created by removing a small *Xba*I/*Nhe*I fragment from the linker region in pXDHXR-L. *S. cerevisiae* GPY55-15Bα was separately transformed with plasmids pXRXDH-S, pXDHXR-S, pXDHXR-M, and pXDHXR-L.

Expression of Artificial Enzyme Complexes with Heterologous Subunit Interactions

S. cerevisiae, strain GPY55-15Bα, was first transformed with plasmid pXDHXR-S. This strain, G-XDHXR-S, was further transformed with plasmid pMW104 containing the *XYL1* and *XYL2* genes encoding native monomers of XR and XDH, respectively, giving strain G-104/XDHXR-S.

Preparation of Crude Cell Extract

Transformants were grown in 200 ml SC selection medium containing 20 g/L glucose in a 1-L baffled flask at

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30 ° C with shaking until midlogarithmic phase. Transformants G-XRXDH-S, G-XDHXR-S, G-XDHXR-M, and G-XDHXR-L and reference strain G-YEpPGK were grown in SC-Ura medium. Transformant G-104/XDHXR-S and reference strain G-104/YEpPGK were grown in SC-Ura-Leu medium containing 20 g/L glucose. The cells were harvested by centrifugation for 10 min at 5000 rpm (Beckman JA-10 rotor) and washed in disintegration buffer [50 mM MOPS (pH 7.0), 1 mM dithiothreitol, 1 mM EDTA]. The pellet was resuspended in 10 ml disintegration buffer containing protease inhibitors (complete protease inhibitor cocktail, Amersham Pharmacia Biotech, Norden AB, Sollentuna, Sweden) and vortexed twice for 5 min at 4 ° C with an equal volume of glass beads (0.5 mm). The disintegrated cell mixture was centrifuged for 15 min at 13,000 rpm (Heraeus microcentrifuge) at 4 ° C and the supernatant was filtered and used directly or stored at −70 ° C until analyzed for size distribution, protein concentration, and enzyme activities.

Size Distribution of Artificial Enzyme Complexes

Gel filtration for determination of size distribution of native enzyme complexes was carried out at 4 ° C on a fast protein liquid chromatography (FPLC) system (Amersham Pharmacia Biotech). A Hiload 16/60 Superdex 200 gel-filtration prepacked column (Amersham Pharmacia Biotech), equilibrated with 20 mM MOPS (pH 7.0), 1 mM dithiothreitol, and 0.150 M NaCl, was used to separate the protein complexes containing XR and XDH activity. The complexes were eluted with buffer at a flow rate of 1.0 ml/min and monitored at 280 nm. Fractions of 0.7 ml were collected and the enzyme elution profiles were determined.

Molecular Weight Determination

Molecular weights of fusion proteins were determined by SDS–PAGE using low-molecular-weight standards (Amersham Pharmacia Biotech) containing phosphorylase *b* (94.0 kDa), bovine serum albumin (BSA, 67.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (30.0 kDa), and α-lactalbumin (14.4 kDa). For molecular weight analysis of native protein complexes, determined by gel filtration, a high-molecular-weight calibration kit (Amersham Pharmacia Biotech) containing ovalbumin (43.0 kDa), aldolase (158.0 kDa), catalase (232 kDa), ferritin (440.0 kDa), thyroglobulin (669 kDa), and Blue Dextran 2000 was used.

Enzyme Activity Assay

XR activity was measured spectrophotometrically by following the oxidation of NADPH at 340 nm. Reaction conditions were 0.1 M triethanolamine (pH 7.0), 0.35 M xylose, and 0.2 mM NADPH (Smiley and Bolen, 1982). XDH activity was measured by monitoring the reduction of NAD^+ at 340 nm in a reaction mixture of the following composition: 0.1 M glycine (pH 9.0), 1 mM $MgCl_2$, 0.3 M xylitol, and 3 mM NAD^+ (Rizzi *et al*., 1989). An extinction coefficient of 6.22 $mM^{-1}\,cm^{-1}$ was used to calculate the activity in units/ml where 1 unit equals the conversion of 1 mmol of substrate per minute. The specific activities of the enzymes were expressed as millimoles of converted substrate per milligram of total protein per minute, equivalent to units per milligram of protein. Protein concentration was measured according to Bradford (1976), with BSA as standard (Bio-Rad, Sweden).

Western Blot Analysis

Protein samples of 10 mg were mixed with loading buffer (1:4) containing 62.5 mM Tris–HCl (pH 6.8), 10% (v/v) glycerol, 10% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, and 1% (w/v) bromophenol blue. After 5 min at 98 ° C the samples were loaded into a 12.5% SDS–PAGE (Laemmli, 1970). After gel electrophoresis, proteins were transferred onto a PVDF membrane (Bio-Rad, Trans-Blot Transfer medium) and Western blot analysis was carried out using xylose-reductase-specific polyclonal antibodies (Hallborn *et al*., 1991) and the alkaline phosphatase method of Bio-Rad (Immunoblot AP system). Marker proteins were visualized by staining with Coomassie blue R-250 (Sigma).

Growth on Xylose Plates

Reference strain G-YEpPGK and transformants G-XRXDH-S, G-XDHXR-S, G-XDHXR-M, and G-XDH XR-L were incubated on SC-Ura plates and G-104 was incubated on SC-Leu plates containing 20 g/L glucose and 20 g/L agar for 3 days at 30 ° C before plating on SC plates containing 20 g/L xylose.

Fermentation Conditions and Analytical Methods

For each set of fermentations a new inoculum from the frozen stock was incubated at 30 ° C for 3 days on plates containing 6.7 g/L yeast nitrogen base supplemented with amino acids, 20 g/L glucose, and 20 g/L agar. Liquid inoculum was grown in two stages. First, 50 ml SC selection medium containing 20 g/L glucose in a 250-ml baffled flask was inoculated from a plate and incubated under orbital shaking, 140 rpm, at 30 ° C, for 24 h. The preculture was then transferred to 250 ml SC selection medium containing 20 g/L glucose in a 1-L baffled flask and incubated as above until the late exponential growth phase was reached. The cells were collected by centrifugation in a Beckman centrifuge (JA-10 Rotor) at 5000 rpm for 15 min. The cells were

washed once with 0.9% NaCl solution and resuspended in SC medium containing 80.0 g/L xylose to an initial cell mass concentration of 10.0 g/L dry wt. Twenty-milliliter flasks were filled with 15 ml SC medium (pH 5.5) containing 80 g/L xylose. The flasks were sealed with rubber stoppers equipped with a cannula to allow gas outflow. Agitation was achieved with a magnetic stirrer. Fermentation was performed at 30° C. Concentrations of sugar substrates and fermentation products were determined using high-performance liquid chromatography (Beckman, Fullerton, CA) with two columns (Aminex Ion-Exclusion HPX-87H, Bio-Rad, Richmond, CA) in series (Lindén and Hahn-Hägerdal, 1989) at 45° C with 5 mM H_2SO_4 as the mobile phase. The compounds were detected with a refractive-index detector [RID-6A differential refractometer (Shimadzu, Kyoto, Japan)].

RESULTS

Molecular Mass Determination

After transformation, production of recombinant fusion proteins in strains G-XRXDH-S, G-XDHXR-S, G-XDHXR-M, G-XDHXR-L, and G-104/XDHXR-S was confirmed by Western blot analysis using xylose-reductase-specific polyclonal antibodies (Fig. 1). The molecular weights of the fusion proteins were estimated to be approximately 75 kDa according to Western blot (Hallborn *et al.*, 1991; Kötter *et al.*, 1990). The theoretical molecular weights of the fusion proteins are 74.86 kDa (pXRXDH-S), 74.79 kDa (pXDHXR-S), 75.13 kDa (pXDHXR-M), and 75.67 kDa (pXDHXR-L) when the molecular weights for XR (Hallborn *et al.*, 1991) and XDH (Kötter *et al.*, 1990) are combined with the molecular weights of the linker region. Protein bands corresponding to native XR were not seen in the Western blot analysis of strains G-XRXDH-S, G-XDHXR-S, G-XDHXR-M, and G-XDHXR-L. Both fusion protein and native XR were observed in strain G-104/XDHXR-S.

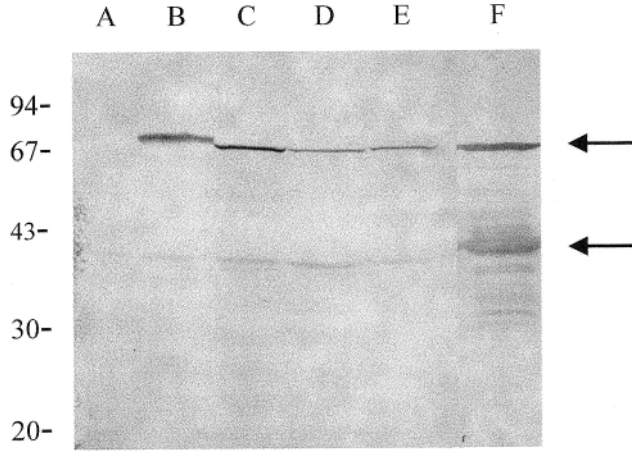

FIG. 1. Western blot analysis of fusion protein and XR produced by *S. cerevisiae* analyzed with Western blotting using xylose-reductase-specific polyclonal antibodies. Lane A, strain G-YEpPGK; lane B, strain G-XRXDH-S; lane C, G-XDHXR-S; lane D, strain G-XDH-XR-M; lane E, strain G-XDHXR-L; lane F, strain G-104/XDHXR-S. Size of the molecular weight markers (in kiloDaltons) is shown on the left. The arrows indicate the positions of fusion proteins and XR monomers.

XR and XDH Activity

Strain G-XRXDH-S showed low XR and XDH activity (0.012 and 0.15 U/mg, respectively). The XDH activity was reduced 40 times compared with the control strain G-104/YEpPGK (XR, 4.3 U/mg; and XDH, 6.20 U/mg), expressing native XR and XDH monomers (Table 2). The activity of XR was similar to the activity in reference strain G-YEpPGK (XR, 0.016 U/mg). When the 5′-end of *XYL1* was fused to the 3′-end of *XYL2* giving plasmid pXDHXR-S, the transformed strain G-XDHXR-S showed both XR (0.11 U/mg) and XDH (0.20 U/mg) activities (Table 2).

The contribution of the linkers to the activities of the fusion proteins was investigated with linkers of different lengths and characters. The specific activities of XDH in strains G-XDHXR-M and G-XDHXR-L increased compared to strains G-XRXDH-S and G-XDHXR-S. Strains G-XDHXR-M and G-XDHXR-L exhibited XDH activities corresponding to 0.36 and 0.46 U/mg, respectively, while strains G-XRXDH-S and G-XDHXR-S only exhibited activities of 0.15 and 0.20 U/mg, respectively (Table 2). The specific XR activities were 0.012 U/mg for strain G-XRXDH-S, 0.11 U/mg for strains G-XDHXR-S and G-XDHXR-L, and 0.17 U/mg for strain G-XDHXR-M.

To increase the activity in the fusion protein, the artificial fusion protein and the native subunits were coproduced in the same cell (Lindbladh, 1992). The specific activities of XR and

TABLE 2

***In Vitro* Enzyme Activities of XR and XDH for Recombinant Yeast Strains**

Strain	Sp act[a] XR	Sp act[a] XDH	XR:XDH
G-YEpPGK	0.016 ± 0.006	nd[b]	—
G-104/YEpPGK	4.30 ± 0.29	6.20 ± 0.32	0.69
G-XRXDH-S	0.012 ± 0.005	0.15 ± 0.05	—
G-XDHXR-S	0.11 ± 0.01	0.20 ± 0.09	0.55
G-XDHXR-M	0.17 ± 0.07	0.36 ± 0.13	0.47
G-XDHXR-L	0.11 ± 0.05	0.46 ± 0.18	0.24
G-104/XDHXR-S	4.15 ± 0.30	5.90 ± 0.40	0.70

Note. Values are presented as means of four independent measurements.

[a] Sp act, specific activity in unit mg protein^{-1}.

[b] nd, not detectable.

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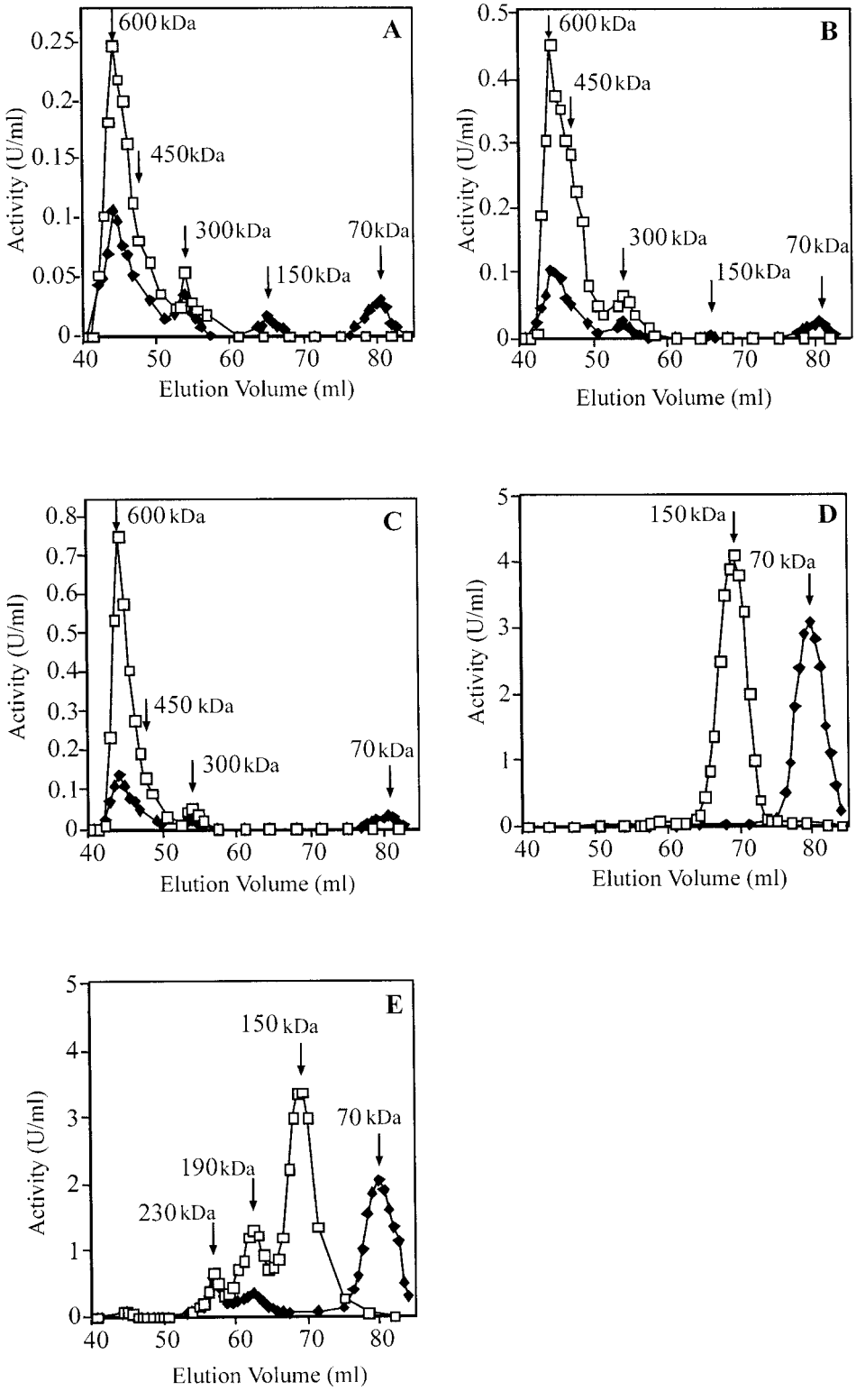

FIG. 2. XR ◆ and XDH □ activity profiles obtained after gel-filtration chromatography on a Hiload 16/60 Superdex 200 column. Fraction volumes were 0.7 ml and the first 40 ml was collected separately. (A) G-XDHXR-S, (B) G-XDHXR-M, (C) G-XDHXR-L, (D) G-104/YEpPGK, and (E) G-104/XDHXR-S. The arrows indicate the elution positions of different protein complexes with XR and XDH activities.

XDH in this strain, G-104/XDHXR-S, cotransformed with plasmids pMW104 and pXDHXR-S (XR, 4.15 U/mg; and XDH, 5.90 U/mg), were similar to those of reference strain G-104/YEpPGK (XR, 4.3 U/mg; and XDH, 6.20 U/mg) (Table 2).

Enzyme Complexes

To verify the existence of functional fusion protein complexes exhibiting both XR and XDH activities, crude extracts from strains G-XDHXR-S, G-XDHXR-M, G-XDHXR-L, and G-104/XDHXR-S and reference strain G-104/YEpPGK were analyzed by gel filtration. Fusion proteins expressed in strains G-XDHXR-S, G-XDHXR-M, and G-XDHXR-L eluted as three partly overlapping peaks corresponding to molecular weights of 600, 450, and 300 kDa (Figs. 2A–2C). The subunit weight of the fusion protein estimated from Western blot analysis is 75 kDa, suggesting that the fusion protein complexes were present in octameric, hexameric, and tetrameric forms. No complexes of higher molecular weights were found. The elution profile of G-XDHXR-S also revealed protein complexes with a molecular weight of 150 kDa, equivalent to dimers (Fig. 2A). This complex exhibited only XR activity, indicating that XDH required more than two subunits for activity. This protein complex was not seen in the elution profiles of G-XDHXR-M and G-XDHXR-L. In the 600-kDa complex the ratio between the XR and XDH activities decreases when longer linkers were used (Figs. 2A–2C). The ratio of XR to XDH in this complex was 0.55 in G-XDHXR-S, 0.47 in G-XDHXR-M, and 0.24 in G-XDHXR-L.

A protein with a molecular weight of 72 kDa showing only XR activity was found in all strains expressing fusion proteins (Figs. 2A–2C). This protein could be a proteolytic product of the fusion protein and/or an unspecific aldo–keto reductase (Kuhn *et al*., 1995).

Crude extract from strain G-104/XDHXR-S, coexpressing fusion protein with native XR and XDH monomers, revealed that monomers of XR and XDH were able to interact with the fusion protein to form enzyme complexes of approximately 230 and 190 kDa and none or very few octameric, hexameric, or tetrameric complexes (Fig. 2E). The production of XR and XDH monomers prevents the formation of higher-order aggregates of the fusion protein. Native dimeric XR and tetrameric XDH with molecular weights of 72 and 154 kDa, respectively, were detected separately from the fusion protein complex (Fig. 2E).

The total activity of 190- and 230-kDa complexes, calculated from the activity profile obtained after gel filtration (Fig. 2E), exhibited 10 and 9 times higher XR and XDH activity, respectively, compared with the original conjugates consisting of only chimerical subunits produced in strain G-XDHXR-S (Fig. 2A). The XR:XDH activity ratio was lower in the 190-kDa complex than in the 230-kDa complex, 0.27 and 0.88, respectively.

Xylose Fermentation

To investigate the influence of the expression of fusion proteins on product formation, xylose fermentation was evaluated under oxygen-limited conditions (Table 3). Strains G-XDHXR-S, G-XDHXR-M, G-XDHXR-L, and G-104/XDHXR-S and reference strains G-YEpPGK and G-104/YEpPGK were cultivated in SC minimal medium containing 80 g/L xylose at 30 °C. Under the chosen conditions—10 g/L dry wt of cells growth was negligible.

Strains G-XDHXR-M and G-XDHXR-L exhibited very low xylose consumption rates and the main product was xylitol (data not shown). Neither ethanol nor glycerol was formed in these strains. Enzyme activity measurements on cells harvested after 6 days of fermentation showed that XR and XDH activities had been completely lost in these cultures (data not shown). Strain G-XDHXR-S consumed 16.16 g/L xylose and formed 3.62 g/L xylitol (Table 3), equivalent to a xylitol yield of 0.22. Ethanol production was 1.06 g/L, equivalent to a yield of 0.066. Strain G-104/XDHXR-S, coexpressing the fusion protein together with native XR and XDH monomers, consumed 49.78 g/L xylose and produced 27.33 g/L xylitol, with a yield of 0.55. Ethanol formation was 3.84 g/L, equivalent to a yield of

TABLE 3

Xylose Consumption and Product Formation, after 8 Days of Incubation in Oxygen-Limited Cultivation with Recombinant Strains in a SC Minimal Medium Containing 80 g/L Xylose

Strain	Xylose (g/L)	Xylitol (g/L)	Glycerol (g/L)	Acetate (g/L)	Ethanol (g/L)	Xylitol yield	Ethanol yield
G-YEpPGK	10.75 ± 0.14	0.89 ± 0.15	0	0	0	—	—
G-104/YEpPGK	57.33 ± 0.39	35.50 ± 0.57	0.09 ± 0.01	3.06 ± 0.01	3.66 ± 0.05	0.62	0.064
G-XDHXR-S	16.16 ± 0.2	3.62 ± 0.23	0.08 ± 0.02	0.64 ± 0.01	1.06 ± 0.08	0.22	0.066
G-104/XDHXR-S	49.78 ± 1.38	27.33 ± 0.46	0.06 ± 0.08	2.46 ± 0.06	3.84 ± 0.01	0.55	0.077

Note. Values are presented as means of three independent cultivations.

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0.077. Reference strain G-104/YEpPGK, expressing only native XR and XDH monomers, consumed most xylose, 57.33 g/L, and produced 35.50 g/L xylitol, with a yield of 0.62, and ethanol formation was only 3.66 g/L, equivalent to a yield of 0.064. Strains G-XDHXR-S and G-104/XDHXR-S produced less acetate than reference strain G-104/YEpPGK (Table 3).

DISCUSSION

S. cerevisiae was metabolically engineered to synthesize artificial fusion proteins composed of the enzymes XR and XDH. The specific activities of XR and XDH were dependent on the order of the enzymes in the hybrid polypeptide chain and the length and the composition of the connecting region. Constructs with XDH at the N-terminus and XR at the C-terminus of the final gene product had functional XR and XDH activities, while construct with XR at the N-terminus and XDH at the C-terminus lacked XR activity. The juxtaposition of XR and XDH in this fusion protein may result in misfolding of the catalytic domain of XR or unfavorable interactions between the chimerical subunits, which may destabilize the complex and affect the quaternary structure. This has previously been reported for a mouse pancreatic α-amylase and *B. subtilis* glucoamylase fusion protein (De Moraes *et al*., 1995) with fusions in both orders.

The linker was important for the XR and XDH activities; the introduction of a longer linker between XR and XDH in the fusion enzyme increased the specific activity of XDH, probably by relaxing intramolecular strains in the fusion protein. In naturally occurring multifunctional enzymes, no homology was observed between the length and the composition of the sequenced linkers (Chang *et al*., 1990; Zalkin *et al*., 1984). Nevertheless, it has been suggested that the correct folding in the polyfunctional AROM enzyme (Purvis *et al*., 1987) tryptophan synthetase (Crawford *et al*., 1987) and in formiminotransferase–cyclodeaminase (Murley and MacKenzie, 1995) depends on the length and composition of the linker joining the functional domains. Similarly, the length of the linker region of several engineered chimerical proteins has proved critical for biological activity. The specific activity of the galactose dehydrogenase part of the *β*-galactosidase–galactose dehydrogenase complex has been increased and the sequential reaction was carried out more efficiently when longer linkers were used as connectors (Carlsson *et al*., 1996). Similar results were obtained for an IL-2 and IL-6 lymphokine fusion protein (Rock *et al*., 1992) and in human granulocyte/macrophage colony-stimulating factor and interleukin-3 fusion protein (Curtis *et al*., 1991). The opposite effect has been reported for a metallothionein fusion protein (Rhee *et al*., 1990) where the level of the activity decreased with the length of the linker.

When native XR and XDH subunits and corresponding fusion proteins were expressed in the same cell, we were able to produce smaller complexes, 190 and 230 kDa, than when only the artificial fusion protein was expressed. The expression of XR and XDH monomers prevented the formation of higher order aggregates and promoted more favorable interactions between the monomers in the fusion protein. The total relative XR and XDH activities of the 190- and 230-kDa fusion protein complexes were increased 10 and 9 times, respectively, compared with the activity of the original fusion protein complexes produced in the G-XDHXR-S strain. The 230-kDa complex had a higher XR:XDH ratio than complexes composed of fusion proteins only. The ratio may reflect unfavorable interactions in complexes consisting of only fusion proteins which was more pronounced for XR and therefore affected its enzymatic activity more. A similar approach has been used to create *β*-galactosidase/galactose dehydrogenase enzyme complexes with 30% higher galactose dehydrogenase activity than in the fusion enzyme (Lindbladh, 1992).

The 230-kDa enzyme complex seemed to be composed of one subunit of native XR and three subunits of native XDH attached to each fusion protein with a deduced molecular weight of 226.5 kDa. Another possibility is that the complex is composed of two native XDH monomers attached to two fusion proteins or a mix of both complexes. The 190-kDa enzyme complex could be a mixture of complexes composed of either one subunit of native XR and two subunits of native XDH or no subunit of native XR and three subunits of native XDH, attached to each fusion protein with deduced molecular weights of 188 and 190.5 kDa, respectively.

Oxygen-limited xylose fermentation by strain G-104/XDHXR-S, coexpressing the fusion protein together with native XR and XDH monomers, produced less xylitol and more ethanol than strain G-104/YEpPGK, expressing only native XR and XDH monomers. The XR and XDH activities in the two strains were essentially the same. The strain expressing the fusion protein produced less acetic acid and less glycerol indicating reduced consumption of NADPH in the XR reaction at the expense of increased consumption of NADH. The proximity generated by the fusion protein probably enhanced the local concentration of NADH around XR so that NADH consumption in the XR reaction increased. Thus, more NAD^+ became available for the XDH reaction, which together with an enhanced local concentration of xylitol increased the flux through the XDH reaction and reduced the secretion of xylitol. The slightly enhanced ethanol formation is the combined result of an increased flux of xylitol through the XDH reaction and reduced loss of carbon as glycerol and acetate.

Proximity effects due to high local concentrations of intermediates have earlier been demonstrated in *E. coli* cells

containing a scavenger enzyme, galactose dehydrogenase, competing with the galactokinase part of a β-galactosidase–galactokinase fusion protein for the galactose formed by β-galactosidase (Carlsson *et al*., 1992). A similar effect was obtained when fused citrate synthase and malate dehydrogenase were expressed in mutants of *S. cerevisiae* deleted in citrate synthase and malate dehydrogenase genes (Elcock and McCammon, 1996; Lindbladh *et al*., 1994).

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