ELSEVIER

Journal of Chromatography B, 737 (2000) 195–202

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

D-Xylose metabolism by *Candida intermedia*: isolation and characterisation of two forms of aldose reductase with different coenzyme specificities

Peter Mayr, Kaspar Brüggler, Klaus D. Kulbe, Bernd Nidetzky*

Division of Biochemical Engineering, Institute of Food Technology, University of Agricultural Sciences (BOKU), Muthgasse 18, A-1190 Vienna, Austria

Abstract

To study individual enzyme components responsible for the initial step of D-xylose utilisation by the yeast *Candida intermedia*, a two-step protocol has been developed that enables clear-cut separation and isolation of two structurally similar but functionally different aldose reductases (ALRs) in high yield. In the first step, the yeast cell extract is fractionated efficiently by biomimetic chromatography using the dye HE-3B (reactive Red 120) as pseudoaffinity ligand coupled to Sepharose CL-4B. In the second step, optimised high-resolution anion-exchange chromatography using Mono Q yields purified ALR1 and ALR2 in overall yields of 63 and 62%, respectively. ALR1 is strictly specific for NADPH ($2.4 \cdot 10^5\ M^{-1}\ s^{-1}$) whereas ALR2 utilises NADH and NADPH with similar specificity constants of approximately $2–4 \cdot 10^5\ M^{-1}\ s^{-1}$. Both enzymes are dimers with a subunit molecular mass of 36 000 but they differ in p*I* and the number of titratable sulphydryl groups in the native protein. The chromatographic procedure identifies microheterogeneity in recombinant aldose reductase from *Candida tenuis* overexpressed in *Escherichia coli*.

Keywords: *Candida intermedia*; Enzymes; Aldose reductase

1. Introduction

Aldose reductase (ALR) is a major component of the enzyme machinery responsible for D-xylose metabolism in yeasts. It catalyses the first step in the catabolic pathway of D-xylose by reducing the pentose sugar to xylitol. ALR belongs to the aldo/keto reductase (AKR) superfamily [1,2]. AKR members are generally monomeric proteins with a molecular mass of about 36 000 and utilise NADPH but not NADH as coenzyme for reduction of carbonyl groups [3]. The ALRs from yeast show a quite large structural and functional variability. They can be dimers [4–6] or monomers [7,8], and are either strictly specific for NADPH [4,9] or in some cases, use both NADH and NADPH for aldehyde reduction [5–8]. There is compelling evidence supporting a role of the NAD(P)H-dependent ALR in D-xylose reduction under oxygen-limited growth conditions [10].

The multiplicity of ALRs in yeast has another interesting facet. While many yeast strains produce one single ALR, which can be NADPH-dependent or NAD(P)H-dependent, there are few organisms such as *Pachysolen tannophilus* which produce two ALRs

*Corresponding author. Tel.: +43-1-36006-6274; fax: +43-1-36006-6251.

E-mail addresses: nide@edv2.boku.ac.at (B. Nidetzky), h8940287@edv2.boku.ac.at (P. Mayr)

0378-4347/00/$ – see front matter
PII: S0378-4347(99)00380-1

with different coenzyme specificity at the same time [11,12]. The simultaneous occurrence of multiple forms of ALR is not well understood in terms of its physiological function [13]. A possible way to advance knowledge in this area would be to characterise individual ALR components with regard to kinetic parameters and substrate specificities. These properties are expected to reflect the role of an enzyme under physiological reaction conditions, at least to some extent. Since ALRs are capable of converting a wide variety of substrates with an aldehyde as the functional group to the corresponding primary alcohols, they could, in principle, serve functions in aldehyde metabolism other than D-xylose reduction [14]. A detailed substrate spectrum approach requires in the first place however, that methods be available enabling the isolation of different ALRs in high purity and yield.

The present paper is concerned with the ALR system of the D-xylose-metabolising yeast *Candida intermedia*. By using a very efficient and simple two-step protocol, it reports purification of two ALRs with different coenzyme specificities. Characterisation of individual enzymes has revealed a number of structural and functional differences between these two ALRs. The purification protocol described in the paper has been used to identify microheterogeneity in preparations of recombinant ALR from *C. tenuis*.

2. Experimental

2.1. Materials

Materials for gel chromatography and electrophoresis were from AmershamPhamacia (Uppsala, Sweden). The dyes Procion Red HE-3B (Reactive Red 120) and Procion Red H8-BN (Reactive Red 31) were obtained from ICI (Manchester, UK). All other chemicals were of the highest purity available.

2.2. Micro-organisms and preparation of the cell-free crude extract

The yeast *C. intermedia* HA409 was obtained from a yeast culture collection maintained at the Institute of Applied Microbiology (BOKU, Vienna, Austria). It was cultivated at 25°C in shaken Erlenmeyer flasks at 130 rpm with YPX medium containing 20 g/l D-xylose, 4 g/l yeast extract and 4 g/l casein peptone (pancreatic digest). After approximately 24 h of growth, cells (typically 20–25 g of wet biomass/l) were harvested by centrifugation (8000 *g*, 15 min, 4°C), washed twice with saline (8.7 g/l NaCl and 0.12 g/l $MgSO_4 \cdot 7H_2O$) and suspended in doubly distilled water (1:3, w/v). The cells were disrupted by three passages through a French pressure cell (1.38 bar, 4°C), then cell debris were removed by ultracentrifugation (100 000 *g*, 35 min, 4°C) and the recovered supernatant (crude cell extract) was stored at −70°C. Any turbidity present after thawing was removed again by ultracentrifugation using the same conditions.

Recombinant aldose reductase from *C. tenuis* CBS4435 was overexpressed in *Escherichia coli* BL21 (DE3) by using the plasmid vector pBeact.li, kindly provided by B. Häcker (Institute of Industrial Genetics, Stuttgart, Germany). As inclusion body formation occurred at higher growth temperatures, the bacterial strain was incubated at 25°C. The medium (pH 7.0) contained 5 g/l D-glucose, 10 g/l tryptone, 5 g/l yeast extract and 5 g/l NaCl. Ampicillin, dissolved in 50% ethanol, was added through a 0.22-μm filter to give a final concentration of 0.1 mg/ml. When cell density, recorded by absorbance at 600 nm, was about 1.0, the cells were induced with 0.5 m*M* isopropyl-β-D-thiogalactopyranosiol (IPTG). Approximately 12 h after the induction, cells (typically 5–10 g wet biomass/l) were harvested by centrifugation (8000 *g*, 15 min, 4°C), and the crude cell extract was obtained as described above.

2.3. Assays and kinetics

All measurements were performed with a Beckman DU-650 spectrophotometer. ALR activity was assayed at 25°C by monitoring the oxidation of NAD(P)H at 340 nm (1–5 min, rate of 0.05–0.1 A/min). One unit of enzyme activity refers to 1 μmol of NAD(P)H consumed/min. All rates were corrected for the appropriate blank readings accounting for the nonspecific oxidation of NAD(P)H such as autooxidation mediated by aldose sugars [15]. The standard reaction mixture contained 50 m*M* potas-

sium phosphate buffer (pH 7.0), 707 m*M* D-xylose, 220 μ*M* NAD(P)H. All reactions were started by the addition of coenzyme. Protein was determined spectrophotometrically at 595 nm by using the Bio-Rad protein assay with bovine serum albumin (BSA) as standard (0.1–1.0 mg/ml).

The apparent kinetic constants were determined at 25°C in a 50 m*M* potassium phosphate buffer (pH 7.0). One substrate was varied over a concentration range of approx. 5–10-times K_m. The other substrate was employed at a constant saturating concentration. All kinetic constants were calculated by non-linear regression methods using SigmaPlot (version 4.01).

2.4. Protein purification

All chromatographic experiments were performed at 20–22°C (room temperature).

2.4.1. Dye ligand chromatography

The matrix for the preparation of the affinity gel was Sepharose CL-4B (AmershamPhamacia). For dye–matrix coupling, 1 ml of gel was suspended in 1 ml of dye solution containing 10 mg of dye suspended in 1 ml doubly distilled water. This solution was stirred gently for 5 min before 120 mg NaCl/ml was added. After 30 min incubation under gentle mixing, solid Na_2CO_3 was added to give pH 11.0–11.5, and this solution was shaken gently at 30°C for at least 48 h. The gel was filtered through a Schleicher&Schuell 595 1/2 filter and washed with 2 *M* NaCl and 20% ethanol until the eluate was colourless. All gels were stored in 20% (v/v) ethanol at 4°C until use.

Screening of a suitable dye for purification of ALR was performed following reported procedures [16]. Briefly, 1 ml of gel was suspended in 10 ml of 50 m*M* potassium phosphate buffer, pH 7.0, and centrifuged (2 min, 100 *g*), and the supernatant was removed. This (washing) procedure was repeated three times. About 1 ml crude cell extract (conductivity 2.5 mS/cm) was applied to 1 ml gel and gently mixed for appropriate binding of the ALRs. After centrifugation the supernatant was removed and the gel was washed with 10 ml buffer. Adsorbed protein was eluted with 5 ml 2 *M* NaCl in buffer, pH 7.0.

2.4.2. Dye affinity chromatography

After selection of Red 120 as dye ligand, purification of the ALRs was performed by column chromatography on an AmershamPharmacia Äkta explorer system. The centrifuged cell extract with a conductivity of 2.5 mS/cm was applied to the dye–ligand column (8×2.5 cm; approximately 10–15 mg protein/ml of gel) equilibrated with five column volumes of 50 m*M* potassium phosphate buffer, pH 7.0 (buffer A). Adsorbed protein was eluted at a flow-rate of 2.5 cm/h with a step gradient of 0.5 *M* and 2 *M* NaCl in buffer A. Fractions of 10 ml were collected and the 2 *M* peak containing most of the ALR activity was pooled. Small amounts of ALRs were also found in the 0.5 *M* peak due to co-elution.

2.4.3. Concentration and desalting

The pooled fractions were concentrated by ultrafiltration at 4°C using a stirred Amicon cell 8200 equipped with a PM 10 filter (cut off 10 000). About 30 ml was removed from the cell, transferred to centrifugal concentrators (Macrosep 10k, cut-off 10 000) and centrifuged (Heraeus Omnifuge.2.0RS, rotor 3360) with 4000 *g* at 4°C to obtain a final volume of less than 10 ml. Desalted preparations of ALRs were obtained by gel permeation with a Sephadex G-25 fine HiPrep 26/10 column (AmershamPharmacia) equilibrated with 40 m*M* Tris–HCl, pH 7.2 (flow-rate 15 ml/min). Small amounts of desalted ALR were prepared by gel permeation with Sephadex G-25 medium in PD-10 columns (AmershamPharmacia) equilibrated with buffer according to the instructions of the supplier.

2.4.4. Ion-exchange chromatography

Separation of two ALRs with ion-exchange chromatography was performed on an AmershamPharmacia Äkta explorer system with a Mono Q HR5/5 column equilibrated with 40 m*M* Tris–HCl buffer, pH 7.2. An amount of 10 mg of protein or less was applied to the column. Elution was accomplished with 0.5 *M* NaCl in buffer in a linear gradient from 0 to 0.5 *M* NaCl in 30 ml. The flow-rate was 1 ml/min. Fractions of 2 ml were collected and those containing ALR1 and ALR2, respectively, were pooled and stored at −70°C.

2.5. Electrophoresis

The efficiency of each purification step was controlled by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), non denaturing anionic PAGE (with PhastGel Gradient 8-25 in both cases) and isoelectric focusing (IEF)–PAGE (with PhastGel IEF 3-9) performed on an AmershamPharmacia PhastSystem unit in accordance with the instructions of the supplier. Staining for activity was performed by assaying the polyol-oxidising activity of ALR. The gel was incubated at pH 9.0 with 950 m*M* xylitol as substrate (1 ml), 2.5 m*M* $NADP^+$ as coenzyme employing 5-methyl phenanzine methosulphate (2 mg/ml, 50 μl used) and Nitro Blue Tetrazolium (6 mg/ml, 50 μl used) as electron acceptors.

2.6. Molecular mass

The molecular mass of the ALR subunits was determined by SDS–PAGE using the LMW standard from AmershamPharmacia as reference. The molecular mass of the native enzymes was determined by gel permeation on Superose 12 (30×1 cm) using a fast protein liquid chromatography (FPLC) system from AmershamPharmacia. The Superose 12 column was equilibrated in 50 m*M* potassium phosphate buffer (pH 7.0) containing 0.3 *M* NaCl, and protein elution was performed at a constant flow-rate of 0.5 ml/min. For calibration cytochrome *c* (12 400), myoglobin (17 600), β-lactoglobulin (35 000), egg albumin (43 000), BSA (67 000) and amyloglucosidase (97 000) were used as standard. A 0.1-ml amount of a 2 mg/ml protein solution was applied to the column.

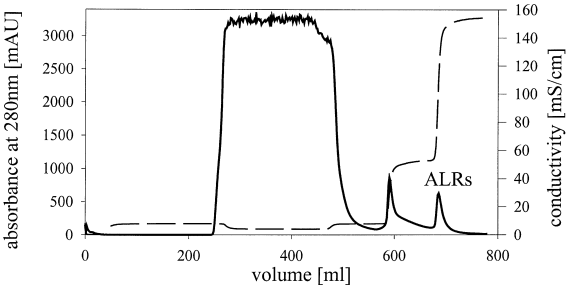

Fig. 1. Purification of ALRs from *C. intermedia* by biomimetic chromatography using Red 120; [conductivity (— — —), absorbance at 280 nm (———)].

2.7. Chemical modification of ALRs

Free thiols in native and fully denatured (0.6%, w/v, SDS treatment) ALRs were determined at pH 7.0 in 10 m*M* potassium phosphate buffer and 25°C with a 10–20-fold excess of Ellman's reagent, NBs_2. A molar coefficient of $13.6 \cdot 10^3$ M^{-1} cm^{-1} at 410 nm for the corresponding NBs_2^- anion was assumed [17].

3. Results and discussion

3.1. Purification

ALR1 and ALR2 have been isolated from cell extracts of *C. intermedia* grown on D-xylose. A summary of the purification is shown in Table 1.

The dye HE-3B (reactive Red 120) has been selected as result of a screening in which the reversible binding of ALR activity to about 20 different dyes differing in overall protein binding capacity was tested. As shown in Fig. 1, ALR

Table 1
Purification of ALR1 and ALR2 from *C. intermedia*

Purification step	Total activity (U)		Specific activity (U/mg)		Purification (fold)		Yield of enzymes (%)	
	ALR1	ALR2	ALR1	ALR2	ALR1	ALR2	ALR1	ALR2
Crude extract	489	187	0.39	0.15	1	1	100	100
RED-120	361	149	8.1	3.3	21	22	74	80
Concentration and desalting	355	136	8.0	2.9	21	22	73	73
Mono Q	310	116	26.8	10.4	69	79	63	62

activity is eluted from the dye column with 2 *M* NaCl while the majority of contaminant protein is either present in the through-fraction or elutes at 0.5 *M* salt. The efficiency of biomimetic chromatography is reflected by an almost 20-fold purification factor obtained in one single step (Table 1). However, ALR1 and ALR2 cannot be separated in this step as reported for other isoenzymes [18,19]. Analysis of the partially purified ALR preparation by IEF–PAGE and staining for ALR activity indicated the presence of two ALR forms with slightly different isoelectric points (p*I*) at around 4.4–4.6. Therefore, anion-exchange chromatography with high resolution was further pursued and optimised by varying the elution buffer (Tris–HCl, His–HCl), the concentration of the buffer (10–40 m*M*), and the pH (6.0–7.2) in order to achieve separation of the enzyme forms, ALR1 and ALR2. No difference in capacity and resolution was found by varying the buffer conditions. Only the recovery of both ALRs increased slightly by about 20% with higher pH and buffer capacity (results not shown). Previously, it has been reported, that use of anion-exchange chromatography with high resolution efficiently separate pancreatic isoenzymes with a Mono S column [20]. Therefore, ion-exchange chromatography by using Mono Q or Mono S columns appears to be a valid method for separating isoenzymes with closely similar physical properties. A typical elution profile from a Mono Q column is shown in Fig. 2, indicating that baseline-separation of the two protein components is obtained under prevailing conditions. In agreement with differences in p*I* values (see below), ALR2 is eluted at a lower salt concentration than ALR1. Judging by the criteria of SDS–PAGE, non denaturing anionic PAGE, IEF–PAGE and analytical gel permeation, the enzymes are pure and can be used for further characterisation. The yields of pure ALR1 and ALR2 are greater than 60%. Problems with stability of ALR activity during anion-exchange chromatography, previously encountered with the ALR from *C. tenuis* in the absence of Tween [7], have not been observed with both ALRs from *C. intermedia*. Inactivation, about 20%, during elution from the Red 120 column with high salt concentrations was unexpected but could be ascribed either to stickiness of ALR1 and ALR2, or instability in the presence of 2 *M* salt. Biomimetic dye–ligand chromatography followed by high-resolution anion-exchange chromatography has been employed in purification of other yeast ALRs, for example from *C. tenuis* [7], *Pichia stipitis* [5], *C. shehatae* [8]. In case of human ALR, this procedure enables the separation of oxidised and nonoxidised protein forms [21]. Therefore, it can be proposed as general strategy to purify ALR enzymes. However, the dye–ligand optimally useful in the first step has to be identified by screening for each individual ALR.

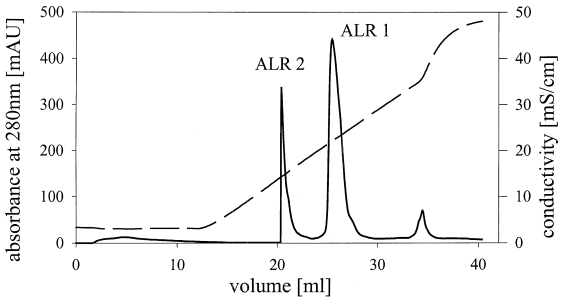

Fig. 2. Purification of ALR1 and ALR2 from *C. intermedia* by anion-exchange chromatography on Mono Q; [conductivity (— — —), absorbance at 280 nm (———)].

3.2. Structural characterisation

On SDS–PAGE, ALR1 and ALR2 migrate as single protein bands with apparent molecular masses of 36 000 (Fig. 3). By using matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF), molecular masses of 35 823 and 35 332 have been determined for ALR1 and ALR2, respectively. Both enzymes appear to be functional dimers. On gel permeation chromatography using Superose HR 12, an identical molecular mass of 58 000 has been determined for ALR1 and ALR2. The calculated molecular mass of the dimer would be 72 000, which is significantly greater than the experimental value. However, interactions of ALR1 and ALR2 with the gel material, probably via hydrophobic bonding, are difficult to avoid completely, even with addition of 0.3 *M* NaCl. Such interactions will lead to underestimation of the true molecular mass of the native protein, and that situation is likely to prevail with both ALRs. The isoelectric points of ALR1 and

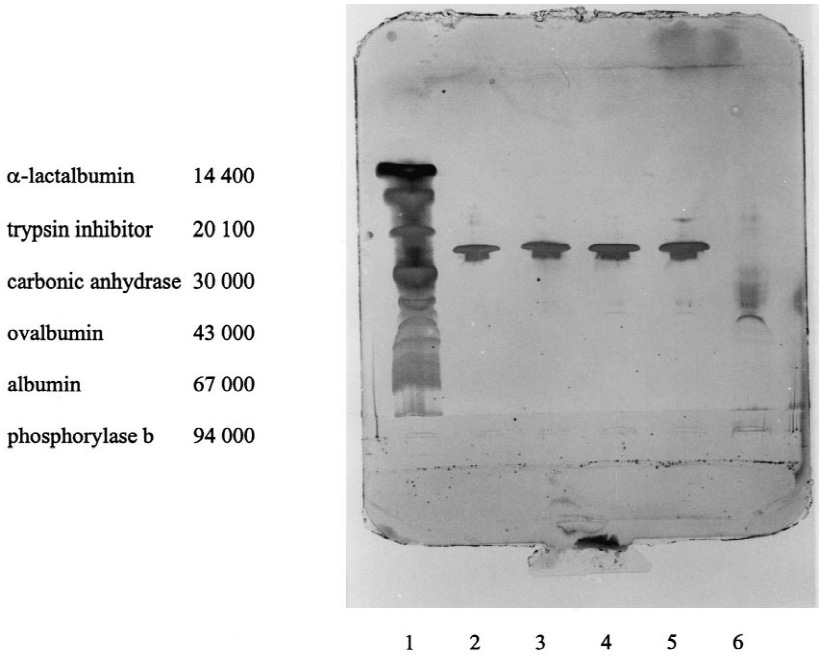

Fig. 3. Purification of ALR1 and ALR2 from *C. intermedia* by SDS–PAGE. Lanes 4–6 other fractions, lane 3 ALR1 after Mono Q, lane 2 ALR2 after Mono Q, lane 1 standards.

ALR2 have been determined by IEF–PAGE, and are 4.38 and 4.59, respectively.

The number of titratable cysteines in ALR1 and ALR2 is quite different as is the effect of cysteine derivatisation on enzyme activity. On colorimetric titration with DTNB, five and seven cysteines are counted in native and SDS-denatured ALR1, respectively. The corresponding values for ALR2 are two and four in the native and SDS-denatured protein, respectively. While cysteines do not appear to be essential in ALR2, oxidation of five cysteines in ALR1 results in a loss of 70% activity, measured at substrate saturation. Thiol oxidation has been an important issue especially in the studies with mammalian ALRs. In human ALR, spontaneous oxidation of a single cysteine, Cys 298, leads to an activated enzyme form that has an about 10-times greater maximum initial velocity of NADPH-dependent aldehyde reduction than the natural enzyme [22]. In ALRs from yeast, oxidation of cysteines seems less important in terms of affecting kinetic properties. However, in *P. stipitis* ALR, which shows activity with both NADH and NADPH, replacement of cysteines by serines using site-directed mutagenesis brings about a two- to three-fold reduction in turnover whereas apparent binding of reactants appears largely unaffected [23].

3.3. Functional characterisation

ALR1 and ALR2 have different coenzyme specificities, and a comparison of the kinetic parameters is shown in Tables 2 and 3. ALR1 is a strictly NADPH-dependent aldose reductase. By contrast, ALR2 is active with NADH and NADPH, and catalytic efficiencies for NADPH-dependent or NADH-dependent reduction of D-xylose are very similar. Notably, yeast ALRs having dual coenzyme specificity generally show a clear preference for NADPH [5,7]. The turnover number of ALR2 with NADH was even greater than that with NADPH. This result indicates that the enzyme/coenzyme interaction contribute to rate limitation of the overall reaction catalysed by ALR2. A second-order rate constant of $2 \cdot 10^5\ M^{-1}\ s^{-1}$ for the reaction of free ALR and free NAD(P)H to give products, lies far below 10^7–$10^9\ M^{-1}\ s^{-1}$ for encounter-limited re-

Table 2
Apparent kinetic constants of ALR1 from *C. intermedia*[a]

Varied substrate	Constant substrate	K_m	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
NADPH	D-Xylose (707 m*M*)	61±8.3 (μ*M*)	14.6±0.7	$2.4 \cdot 10^5$
D-Xylose	NADPH (0.22 m*M*)	82±6 (m*M*)	14.6±0.2	178

[a] Reactions were performed at 25°C in 50 m*M* potassium phosphate buffer, pH 7.0. Calculation was by non linear regression analysis. The constants are k_{cat}, the catalytic constant, and K_m, the Michaelis constant.

Table 3
Apparent kinetic constants of ALR2 from *C. intermedia*[a]

Varied substrate	Constant substrate	K_m	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
NADH	D-Xylose (707 m*M*)	28±1.1 (μ*M*)	11.2±0.12	$4 \cdot 10^5$
NADPH	D-Xylose (707 m*M*)	56±12.8 (μ*M*)	11±1	$2 \cdot 10^5$
D-Xylose	NADH (0.22 m*M*)	50±8 (m*M*)	10.5±0.7	210
D-Xylose	NADPH (0.22 m*M*)	52±4.3 (m*M*)	9.8±0.2	188

[a] Reactions were performed at 25°C in 50 m*M* potassium phosphate buffer, pH 7.0. Calculation was by non linear regression analysis. The constants are k_{cat}, the catalytic constant, and K_m, the Michaelis constant.

actions [24], which is a good indication that NAD(P)H binding to ALR1 and ALR2 is a two-step process. The structural and kinetic basis for ALR/nucleotide interaction has been described for mammalian ALRs [25,26] to which yeast ALRs are structurally related [1]. The kinetic parameters obtained with D-xylose as varied substrate are summarised in Tables 2 and 3. The catalytic efficiencies are remarkably similar for ALR1 and ALR2, and the apparent substrate binding constants (K_m) show little probably insignificant variation. The kinetic data suggest that ALR2 can catalyse D-xylose reduction with similar efficiencies by using either NADH or NADPH as coenzyme. The ratio of available NADH to NADPH that is prevailing in the yeast cytosol will determine whether NADH or NADPH is utilised by ALR2.

3.4. Purification of recombinant ALR from C. tenuis

The gene encoding ALR from *C. tenuis* has been overexpressed in *E. coli*, and soluble ALR has been purified by using the procedure reported previously [7]. Results are summarised in Table 4. On anion-exchange chromatography on Mono Q, significant microheterogeneity of the protein fraction containing ALR activity has been detected (Fig. 4). The major fraction (6) has a p*I* of 4.98–5.05. Fraction 8 which elutes last has a p*I* of 4.66–4.72. The specific activities of fractions 7 and 8 are approx. 30% smaller than that of the major ALR fraction. The ratios of NADPH- to NADH-linked enzyme activities are similar with individual enzyme fractions (Table 4). Although it remains unclear which effects

Table 4
Purification of ALR from *C. tenuis* expressed in *E. coli* BL21 (DE3)

Purification step	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield of enzyme (%)	Ratio NADPH/NADH
Crude extract	910	1.15	1	100	2
Red 31	720	6.21	5.4	79	2
Concentration and desalting	720	6.21	5.4	79	2
Mono Q fraction 6	466	19.3	16.8	51	1.9
Mono Q fraction 7	144	13.5	11.7	15.8	1.8
Mono Q fraction 8	44	12.1	10.5	4.8	1.9

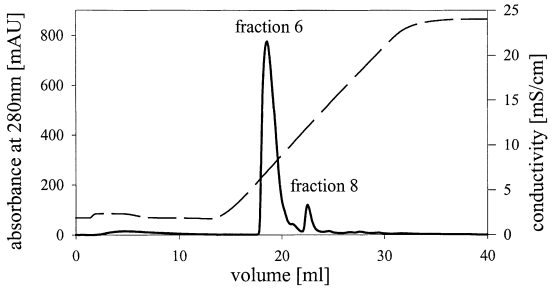

Fig. 4. Purification of ALR from *C. tenuis* expressed in *E. coli* BL21 (DE3) by anion-exchange chromatography on Mono Q; [absorbance at 280 nm (———), conductivity (— — —)].

have been responsible for creating microheterogeneity in recombinant ALR, the demonstration of the occurrence of microheterogeneity is of course important in view of future applications of the enzyme [27] as well as for improving the overexpression.

Acknowledgements

The authors acknowledge the financial support by grants from the European Union (EC FAIR CT 1048) and from the Austrian Science Foundation (P-12569-MOB).

References

[1] H. Lee, Yeast 14 (1998) 977.
[2] K.M. Bohren, B. Bullock, B. Wermuth, K.H. Gabbay, J. Biol. Chem. 264 (1989) 9547.
[3] J.M. Jez, M.J. Bennett, B.P. Schlegel, M. Lewis, T.M. Penning, Biochem. J. 326 (1997) 625.
[4] S.I. Yokoyama, T. Suzuki, K. Kawai, H. Horitsu, K. Takamizawa, J. Ferment. Bioeng. 79 (1995) 217.
[5] C. Verduyn, R. van Kleef, J. Frank, H. Schreuder, J.P. van Dijken, W.A. Scheffers, Biochem. J. 226 (1985) 669.
[6] S. Kise, N. Koizumi, H. Maeda, J. Ferment. Technol. 66 (1988) 615.
[7] W. Neuhauser, D. Haltrich, K.D. Kulbe, B. Nidetzky, Biochem. J. 326 (1997) 683.
[8] N.W.Y. Ho, F.P. Lin, S. Huang, P.C. Andrews, G.T. Tsao, Enzyme Microb. Technol. 12 (1990) 33.
[9] G. Ditzelmüller, C.P. Kubicek, W. Wöhrer, M. Röhr, Can. J. Microbiol. 30 (1984) 1330.
[10] J.P. van Dijken, W.A. Scheffers, FEMS Microbiol. Rev. 32 (1986) 199.
[11] G. Ditzelmüller, E.M. Kubicek-Pranz, M. Röhr, C.P. Kubicek, Appl. Microbiol. Biotechnol. 22 (1985) 297.
[12] C. Verduyn, J. Frank, J.P. van Dijken, W.A. Scheffers, FEMS Microbiol. Lett. 30 (1985) 313.
[13] H. Schneider, H. Lee, M.F.S. de Barbosa, C.P. Kubicek, A.E. James, Appl. Environ. Microbiol. 55 (1989) 2877.
[14] C.E. Grimshaw, Biochemistry 31 (1992) 10139.
[15] S.P. Wolff, M.J.C. Crabbe, Biochem. J. 226 (1985) 625.
[16] R.K. Scopes, J. Chromatogr. 376 (1986) 131.
[17] A.F.S.A. Habeeb, Methods Enzymol. 25 (1972) 457.
[18] J. Kirchberger, M.A. Vijayalakshmi, G. Kopperschläger, J. Chromatogr. 557 (1991) 325.
[19] J. Kirchberger, U. Domar, G. Kopperschläger, T. Stigbrand, J. Chromatogr. 574 (1992) 237.
[20] M.J. Fuchs, V. Keim, J. Chromatogr. 576 (1992) 287.
[21] C.E. Grimshaw, Arch. Biochem. Biophys. 278 (1990) 273.
[22] C.E. Grimshaw, M. Shabbaz, G. Jahangiri, C.G. Putney, S.R. McKercher, E.J. Mathur, Biochemistry 28 (1989) 5343.
[23] Y. Zhang, H. Lee, FEMS Microbiol. Lett. 147 (1997) 227.
[24] A.R. Fersht, Enzyme Structure and Mechanism, W.H. Freeman, New York, 1985.
[25] D.K. Wilson, K.M. Bohren, K. Gabbay, F. Quiocho, Science 257 (1992) 81.
[26] C.E. Grimshaw, K.M. Bohren, C.J. Lai, K.H. Gabbay, Biochemistry 34 (1995) 14356.
[27] B. Nidetzky, W. Neuhauser, D. Haltrich, K.D. Kulbe, Biotechnol. Bioeng. 52 (1996) 387.