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Protein engineering of *Drosophila* alcohol dehydrogenase

The hydroxyl group of Tyr^{152} is involved in the active site of the enzyme

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Drosophila alcohol dehydrogenase is the most studied member of the family of short-chain alcohol dehydrogenases, although its tridimensional structure still remains unknown. We have engineered a *Drosophila* alcohol dehydrogenase in which tyrosine-152, an invariant residue in all members of the family, has been substituted by phenylalanine. The mutated gene has been expressed in yeast and pure mutant enzyme has been prepared by a one-step FPLC chromatographic procedure. *Drosophila* alcohol dehydrogenase-phenylalanine-152 shows no enzymatic activity. This result suggests not only that tyrosine-152 could constitute an essential building block of the active site but also that its hydroxyl group is directly involved in the redox reaction catalyzed by the enzyme.

Site-directed-mutagenesis; *Drosophila*; Alcohol dehydrogenase; Reactive residue

1. INTRODUCTION

Drosophila alcohol dehydrogenase (ADH, alcohol NAD^+ oxidoreductase; EC 1.1.1.1) is the member of the short-chain dehydrogenase family on which the largest amount of genetic, biochemical and structural information has been gathered [1]. Specific roles for several amino acids and some secondary structure predictions have been described for the *D. melanogaster* enzyme [2,3]. However, only indirect evidence is available concerning the residues involved in the active domains of ADH, as its structure is known only at the amino acid sequence level. Very recently, the first crystallographic analysis of a short chain dehydrogenase has been described [4], and the tridimensional prediction for *Drosophila* ADH is being approached through protein modelling. In the meantime, comparison of conserved residues among all short-chain dehydrogenases, and between these and the well-known long-chain ADHs, have provided valuable clues about protein sites involved in the catalytic domains [5]. It has been claimed that the secondary structure of the N-terminal region is organized into a Rossman fold nucleotide-binding domain, consisting of alternating α-helices and β-sheets with highly conserved Gly and Asp residues (Gly^{14}, Gly^{16}, Gly^{19}, Asp^{37}). Site-directed mutagenesis in these amino acids seems to confirm their predicted role in building the correct structure of the NAD^+ binding pocket [6,7]. Yet no data are available about the presumptive substrate interactive region. Primary structure comparisons have shown a highly conserved region in the middle of a polypeptide comprising amino acids Tyr^{152} and Lys^{156}. As short chain dehydrogenases do not require metal ions for their activity, an amino acid side chain has to hold the hydroxyl group in the correct position to mediate the electron transfer to the coenzyme. Biochemical data [8], identification of residues by chemical modification [9] and functional characterization of amino acids by site-directed mutagenesis of the related enzyme human-placental-15-hydroxyprostaglandin dehydrogenase [10] strongly suggest that Tyr^{152} is one of the amino acids involved in the catalytic sites of *Drosophila* ADH. To confirm this hypothesis, we have engineered a *Drosophila* ADH-Phe^{152} enzyme. We have assumed that this conservative change would not alter the overall conformation of this region and therefore changes in enzyme behaviour could not be attributed to structural disruption, but rather to an alteration of a certain chemical group essential for the catalytic function. The mutant has been expressed in a *S. cerevisiae* host/vector system previously described [11] and a one-step purification protocol by FPLC chromatography has been designed. Both crude yeast extracts and pure enzyme preparations have been assayed for ADH activity and protein content.

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2. MATERIALS AND METHODS

2.1. *Materials*

T4 DNA ligase, *Bam*HI, *Hin*dIII, *Xho*I, dNTP's and 3,4-dichloroisocoumarin were obtained from Boehringer. *Spe*I was from New England Biolabs. *Acc*I and the DNA Sequencing Kit were from Pharmacia. Replitherm Thermostable DNA Polymerase Kit was purchased

from Epicentre Technologies. [α-^{35}S]dATP and Hybond-C nitrocellulose filters were from Amersham. PCR primers were synthesized by Oligos Etc. Inc. 1,10-Phenanthroline and E64 were obtained from Sigma. Other reported chemicals and reagents were from Sigma or Merck, and culture media reagents from Difco.

2.2. *Organisms and plasmids*

The following strains were used: *E. coli* JM109 [12] and *S. cerevisiae* WV36-201, an ADH-null strain. *D. melanogaster Adh*s intronless coding sequence was originally cloned in plasmid p3008. The phagemid pVT-U was used to express the *Drosophila Adh* gene in yeast (giving plasmid pVT-Adh). Vectors, plasmid constructions, *S. cerevisiae* strains and *Adh* expression in yeast have been previously described [11].

2.3. *DNA manipulation procedures*

Standard DNA cloning procedures were performed according to Maniatis [13] or following enzyme supplier's instructions. DNA from Minipreps was used for restriction analysis, fragment purification and bacterial transformations. DNA to transform yeast cells by the lithium salt method [14] was prepared using the Qiagen plasmid Kit for Midi preparations. Sequencing of double-stranded DNA with [α-^{35}S]dATP was carried out with the Pharmacia Sequencing Kit.

2.4. *Site-directed mutagenesis*

Site-directed mutagenesis was performed according to a cassette replacement method based on two coupled PCR reactions [15]. The first PCR reaction was carried out using the oligonucleotide 5'GGTGCCGGAGAAGACGGGCA3' as mutagenic primer (1291–1310 fragment of the non-coding strand) and the 20mer 5'TTCTGGACTTCTGGGACAA3' as upstream primer (1201–1220 fragment of the coding strand) (see complete ADH gene sequence in [16]). The second PCR reaction was carried out using the entire purified product of the first PCR as upstream and the 17mer/M13 primer (5'GTAAAACGACGGCCAGT3') as the downstream primer, taking advantage of the fact that the gene is cloned in a pUC derived vector (p3008). The first PCR amplification was performed in a final volume of 100 μl, containing 10 ng of p3008 (template DNA), 100 pmol of each primer, 2.5 μg of Repliterm Polymerase and 200 μM dNTP's. Samples were first kept at 94°C for 120 s to ensure initial denaturation, then 30 cycles of 60 s at 94°C (denaturation), 60 s at 55°C (annealing) and 60 s at 72°C (extension). Finally, 10 min at 72°C ensured that all amplified DNA was double-stranded. Products were fractionated on a 1.8% agarose gel and the band corresponding to the desired fragment was purified. Conditions for the second PCR were those described for the first step, except that the concentration of dNTP's was 400 μM, and the primer extension time was extended to 3 min per cycle, owing to the length of the segment to be amplified. Products of this second PCR were extracted once with phenol/chloroform, once with chloroform/ isoamyl alcohol, precipitated with ethanol/sodium acetate, pH 3.5 (2:0.1 v/v), resuspended in 12 μl of TE and further digested with *Bam*HI and *Xba*I. Plasmid p3008, carrying the wild type *Adh* gene, had been cleaved with the same enzymes so that the PCR fragment carrying the mutated codon could replace the original (p3008-Phe).

2.5. *Yeast cultures and protein extraction procedures*

Yeast clones were kept on yeast omission media (YOM) selective media plates and grown in liquid yeast peptone dextrose (YPD) cultures for expression of the heterologous proteins, as previously described [11]. Small scale crude extracts [11] provided enough material to assay ADH activity and to analyze protein and *Drosophila* ADH content by SDS-PAGE and immunoblotting. ADH was purified from large scale extracts prepared according to a glass beads cell disruption method [17], using as protease inhibitor the following 5 × mix: 20 mM EDTA, 20 mM PMSF, 10 μg/ml each of pepstatin, leupeptin, chymostatin, antipain, 0.5 mM 3,4-dichloroisocoumarin, 0.5 mM 1,10-phenanthroline and 0.1 mM E64C. Protein was quantified by the method of Bradford [18].

2.6. Drosophila *ADH purification*

The supernatant resulting from the disruption of 1 liter of yeast culture was first clarified by the addition of salmine sulfate to a final concentration of 0.28% (w/v) followed by centrifugation at 12,000 rpm for 20 min. The resultant supernatant was then fractionated by $(NH_4)_2SO_4$, and the protein precipitated between 35 and 55% saturation, then resuspended in 20 mM Tris-HCl buffer, pH 8.6, supplemented with 1% 2-propanol and 10^{-4} M DTT. The sample was desalted either by filtration chromatography through a 2.5 × 20 cm Sephadex- G25 column or by overnight dialysis. In the first case, fractions showing ADH activity were pooled and the solution was concentrated 10-fold using Ultrafree-CL filters Millipore (10 kDa exclusion pore). Aliquots of 500 μl of the crude ADH preparation were injected directly to a FPLC gel filtration system: Superose 12 and Superose 6 (Pharmacia) FPLC columns were connected in series to sum their resolution ranges. Columns had been equilibrated with the same Tris-HCl buffer. Samples were run at 0.3 ml/min and protein content was recorded by absorbance at 280 nm UV. Fractions showing ADH activity were kept at 4°C for further analysis.

2.7. Drosophila *ADH activity assay*

Drosophila ADH activity was measured by the increase of absorbance at 340 nm, recorded spectrophotometrically at 25°C using 2-propanol as substrate [19].

2.8. *Electrophoresis and immunoblotting*

SDS-PAGE was performed according to Laemmli [20], in 15% acrylamide gels. Proteins were visualized by both 0.1% Coomassie brillant blue and conventional silver staining. Western blotting was performed with a monoclonal antibody against *Drosophila* ADH (LLBE8) [21], following the procedures described in [22]. Quantification of protein bands on SDS-gels and immunoreacting bands on Western blots was performed by scanning densitometry in an Ultroscan XL equipped with the Gel Scan data processing Software (Pharmacia-LKB).

2.9. *Sequence analysis*

Cloning and sequencing were designed using the Sequence Analysis Software Package of the Genetics Computer Group of the University of Wisconsin (GCG) [23].

3. RESULTS AND DISCUSSION

3.1. *Mutagenesis and plasmid constructs*

Transformed *E. coli* JM109 cells were first screened for the mutant gene (p3008-Phe) by an *Acc*I digestion of their plasmid DNA, on account of the *Acc*I restriction site lost when the Tyr triplet (TAC) was replaced by the Phe triplet (TTC). Positive clones were further analyzed by DNA sequencing of the entire ADH coding region and compared with the same fragment in p3008 (Fig. 1). We thus verified the mutant sequence and established that no additional changes had been produced with respect to the *Adh*^{s} reference gene. A *Hin*dIII–*Spe*I fragment containing the mutated gene was obtained from p3008-Phe and cloned into the *Hin*dIII and *Xba*I sites of pVT-U, as previously described for wild-type *Adh* [11], yielding plasmid pVT-Adh-Phe. Yeast strain WV-201-36 was transformed with this plasmid to analyze ADH-Phe^{152} activity (Y-Phe strain). Positive and negative controls were the same yeast strain transformed with the pVT-Adh (wild-type) plasmid (Y-Wt strain) and the pVT-U plasmid without insert (Y-ϕ strain), respectively. Transformants were isolated by

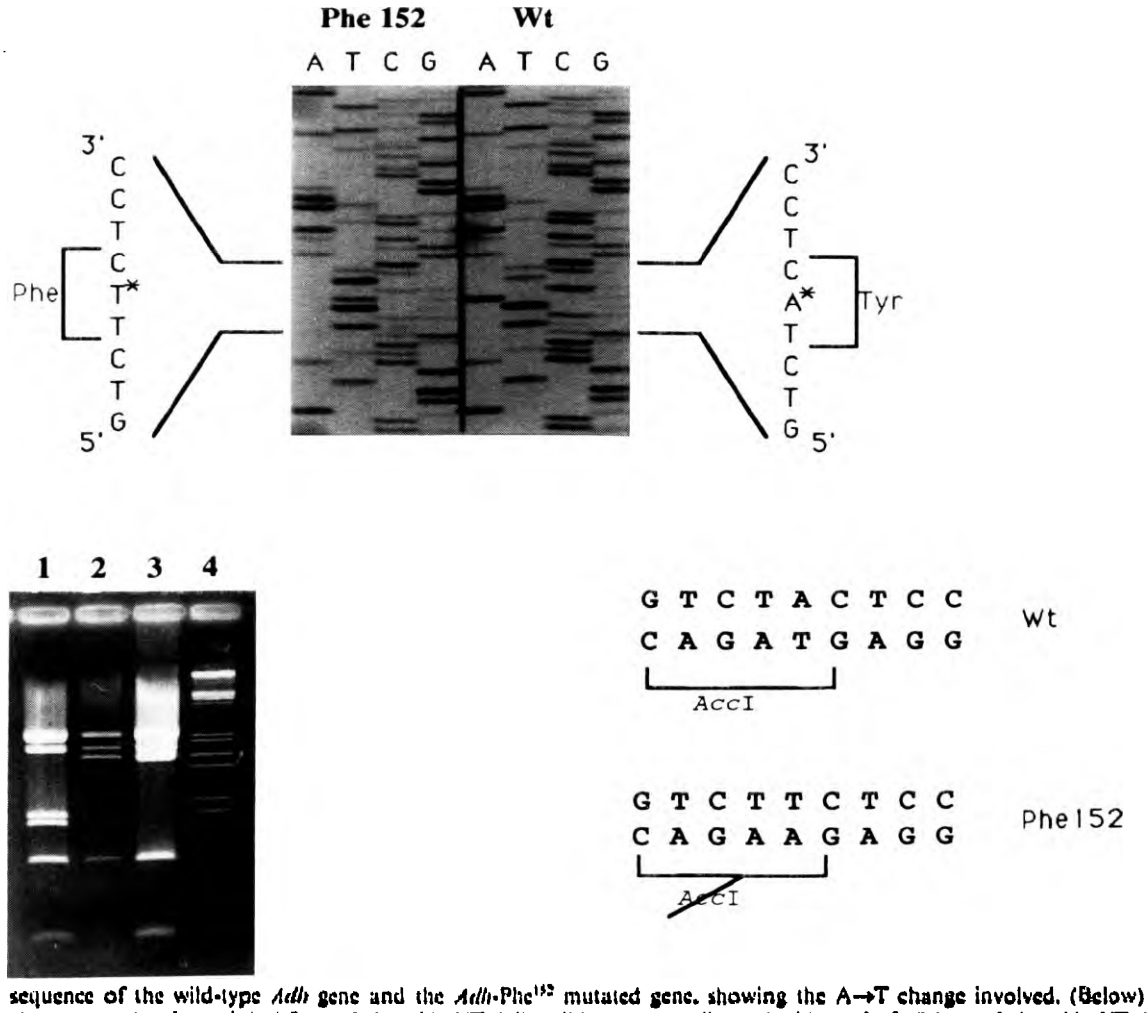

Fig. 1. DNA sequence of the wild-type *Adh* gene and the *Adh*-Phe[152] mutated gene, showing the A→T change involved. (Below) Ethidium bromide-stained agarose gel (0.8%) of (1) 0.5 μg of plasmid pVT-Adh (wild-type gene) digested with *Acc*I, (2) 0.1 μg of plasmid pVT-Adh-Phe[152] digested with *Acc*I, (3) 1 μg of plasmid pVT-Adh-Phe[152] digested with *Acc*I and (4) 150 ng of λDNA digested with *Eco*RI and *Hin*dIII.

their ability to grow on selective media lacking uracil. Standard plasmid rescue procedures and *Acc*I restriction digestion were performed to verify that yeast cells carried the correct construction in each case (Fig. 1).

3.2. *Wild-type and ADH-Phe152 expression*

Crude extracts were prepared from selective 10 ml overnight cultures of Y-φ, Y-Wt and Y-Phe strains and used to quantify ADH activity. Values were considered according to total protein content of the samples, previ-

Table 1

Expression of *Drosophila* ADH in yeast strain WV201-36

Transformant plasmid	Total yeast protein	ADH activity	Specific ADH activity
pVT-φ	1.401 μg/μl[a]	0.001 mU/μl[b]	0.000 mU/μg[c]
pVT-Adh (Wt)	5.420 μg/μl	1.950 mU/μl	0.360 mU/μg
pVT-Phe[152]	1.003 μg/μl	0.001 mU/μl	0.000 mU/μg

[a]Concentration of yeast protein in 100 μl extracted from 15 ml of culture.
[b]mU of ADH activity per minute at 25°C per μg of yeast extract.
[c]mU of ADH activity per μg of total yeast protein.

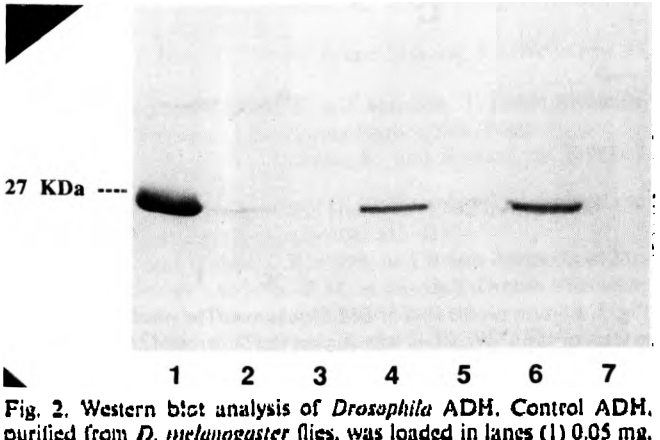

Fig. 2. Western blot analysis of *Drosophila* ADH. Control ADH, purified from *D. melanogaster* flies, was loaded in lanes (1) 0.05 mg, and (2) 1 μg. Protein extracts were from the following yeast strains (3) 20 μl of Y-φ (WV201-36 transformed with pVT-U plasmid without insert), (4) 20 μl of Y-Phe (WV201-36 transformed with pVT-Adh-Phe[152] mutant plasmid), and (6) 5 μl of Y-Wt (WV201-36 transformed with pVT-Adh wild-type plasmid). (5) and (7) are plain sample buffer.

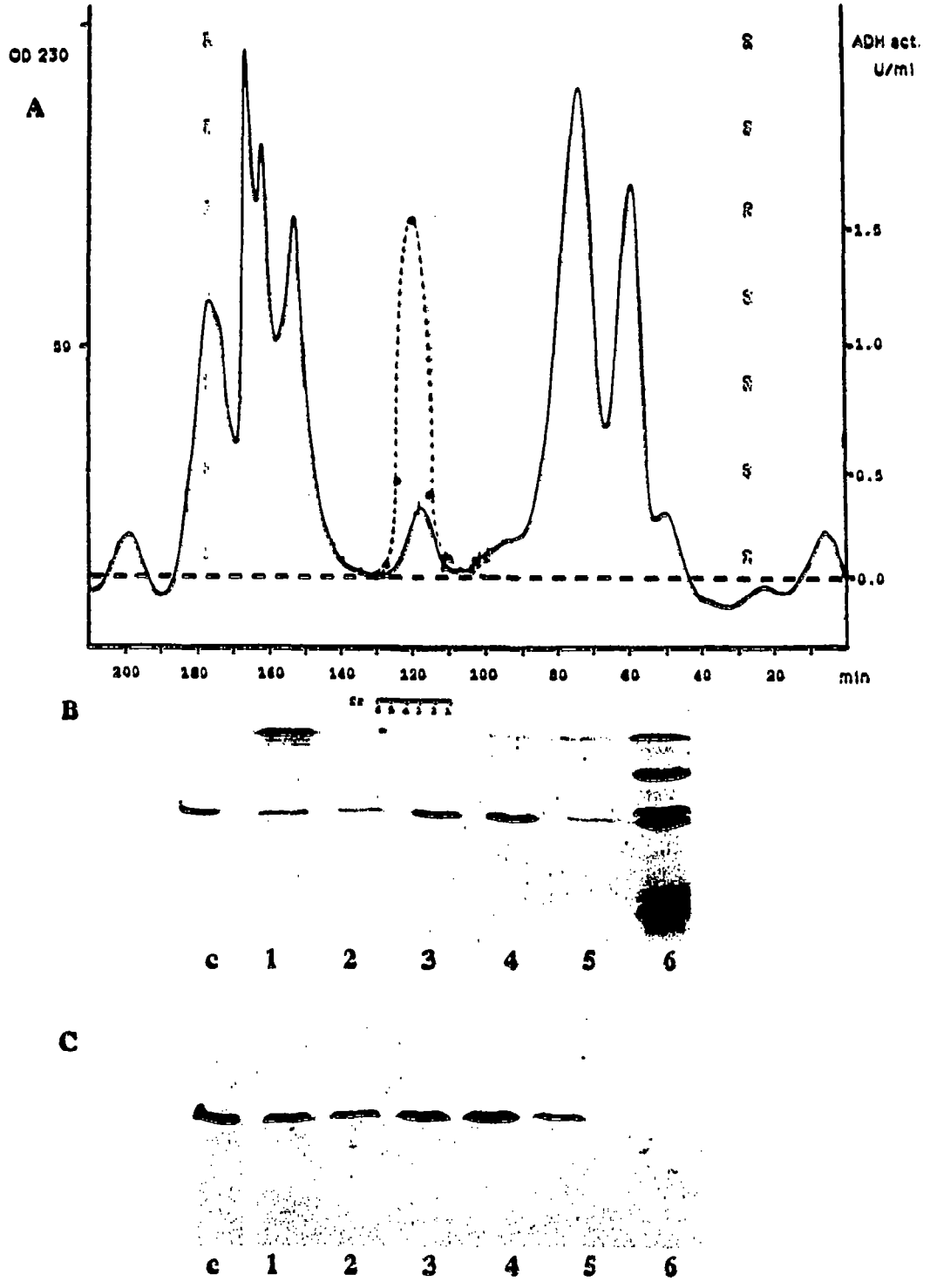

Fig. 3. Elution profile (—) of FPLC columns. The purification rationale was as follows: 500 μl of 35–55% ammonium sulfate-fractionated protein extract of the Y-Wt strain was run on the Superose 12+Superose6 series system and absorbance at 230 nm was recorded. Sample eluted between 110 and 130 min after injection was collected as 1 ml fractions and ADH activity was quantified spectrophotometrically at 340 nm (-·-·-·-). (B) SDS-PAGE of 10 μl aliquots of the fractions collected, stained for total protein with Coomassie blue: 'c' = 5 μg of control ADH (purified from fly homogenates). (1) to (5) aliquots of fractions 1–5, respectively. (6) Molecular weight markers. (C) Immunoblotting of a replica of the previous gel, showing that ADH activity corresponds to Coomassie blue stained bands.

ously quantified following Bradford. As shown in Table I, no significant ADH activity was detected in the Y-Phe extracts. Protein content from Y-ϕ and Y-Phe cultures were similar and rather low compared with that of Y-Wt. This is in agreement with the poor growth rate of these defective strains, which lack any ADH activity,

whereas *Drosophila Adh* gene complements the mutant host [11]. To assess the presence of ADH protein in the Y-Phe extracts, equivalent amounts of total protein of each strain were immunodetected, using *Drosophila* ADH monoclonal antibodies. Western blots (Fig. 2) showed that yeast cells synthesizing mutant ADH produced a similar amount of polypeptide to those expressing the wild-type gene.

3.3. *Yeast-synthesized ADH purification*

In order to extend the analysis of our preliminary results with crude extracts, a one step-purification method was performed with both Y-Wt and Y-Phe extracts from 1 liter cultures. Fig. 3A shows the profile obtained when total yeast protein of Y-Wt was chromatographed by FPLC as described in Materials and Methods. Previous assays with purified preparations of *Drosophila* ADH gave an approximate retention time of 120 min for the active dimer. In addition, fractionation of the yeast crude homogenate produced an isolated peak at the same retention time, which showed the ADH activity expected for the *Drosophila* enzyme. Coomassie blue staining of a SDS-polyacrylamide gel of the positive fractions revealed the presence of the predicted 27 kDa ADH monomer, 95% pure, following densitometry. Western blotting confirmed the ADH nature of the 27 kDa bands (Fig. 3B). When total yeast extracts from Y-Phe were chromatographed under the same conditions, an isolated peak of protein was detected at the corresponding retention time (data not shown). SDS-polyacrylamide gel stained with Coomassie blue and further immunodetected allowed the identification of the expected ADH 27 kDa band, but no enzymatic activity could be recorded in any of these fractions.

3.4. *Behaviour of the mutant ADH-Phe^{152}*

Lack of ADH-Phe^{152} catalytic activity in purified preparations supports the predicted critical role of Tyr^{152} in the substrate-binding pocket of the catalytic domain. Our results further suggest that the hydroxyl group of the Tyr side chain could play an active role in a hydrogen-bond interaction between the substrate and the established enzyme–coenzyme complex during catalysis. Besides, our data agree with those obtained from 15-hydroxyprostaglandin dehydrogenase [10], although no direct correlation could be made between the catalytic domains of the two enzymes, as they bind structurally different substrates. Some authors thus claim that different subclasses of short chain dehydrogenases should be considered [4]. Moreover, the loss of activity in the mutated 15-hydroxyprostaglandin dehydrogenase (Tyr^{151}-Ala) may have been due not only to the absence of the reactive group but also to a structural alteration of the catalytic site, whereas this second explanation could not be invoked in our case. On the other hand, the similarity on the elution profiles of *Drosophila* wild-type ADH and the mutated enzyme seems to suggest that the two can produce apparently similar dimeric forms. Some authors claim that Tyr^{152} could play a critical role in subunit binding to produce active dimeric forms [10], but our results do not seem to support this interpretation. In the absence of a tridimensional structure for *Drosophila* ADH, we postulate that the Tyr^{152} residue lies in the core of the catalytic domain and makes an essential contribution to the electron transfer route, indispensable for substrate transformation.

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