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Preparation and characterization of isozymes and isoforms of horse liver alcohol dehydrogenase

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Abstract

The procedure described allows the simultaneous large-scale preparation of the three main isozymes (EE, ES, SS) of alcohol dehydrogenase from horse liver (HLADH) and their subfractions using heat denaturation, ammonium sulfate precipitation, DEAE and CM ion-exchange chromatography as well as AMP-Sepharose affinity chromatography. Typical yields that can be obtained within three weeks are 1.5–2.5 g of EE-HLADH, 300–800 mg of ES-HLADH, 20–400 mg of SS-HLADH and 50–100 mg of EE-HLADH isoforms from 5 kg of horse liver. The EE-HLADH isoform prepared has a p*I* of 7.8, which is 0.3 pH units lower as compared to the main fraction; the zinc content and number of free sulfhydryl groups are unchanged but matrix-assisted laser desorption ionization mass spectrometry resulted in a molecular mass difference of + 130 to 165 relative molecular mass. From a sugar determination and comparison of its p*I* with an artificial glycosylation product of the EE-HLADH isozyme we concluded that the isoforms of HLADH are non-enzymatic glycosylation products which have been described to occur during protein aging.

1. Introduction

Alcohol dehydrogenases are widespread enzymes which can be found in all organisms. According to their structures they are divided in several lines, families and classes. Until now five classes have been described for mammalians according to their occurrence, substrate specificity and inhibition patterns [1,2]. One of the best-studied dehydrogenases is the class I alcohol dehydrogenase from horse liver (HLADH), a dimeric enzyme that contains two zinc ions per subunit.

Bonnichsen and Wassén [3] described in 1948 the first preparation procedure for HLADH. During the following years it was found that the enzyme preparations were heterogeneous when examined by various fractionation methods [4–7]. On starch gels up to twelve different fractions with enzymatic activity have been observed [8,9]. The three main isozymes of HLADH (EE-, ES-, and SS-HLADH) are formed by dimeric combination of the subunits "E" (Ethanol-active) and "S" (additionally Steroid-active) (for nomenclature see Ref. [10]). The two polypeptide chains E and S show variations in ten out of 374 amino acid positions [11–13], whereas structural variations of the so-called "prime fractions", "subfractions" or "isoforms" have not yet been clarified. It is generally accepted that these minor fractions are derivatives of the main isozymes without underlying genetic differences.

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The following hypotheses for their formation have been discussed: conformational changes of the main fractions [7], disulfide bridge formation as a consequence of oxidation [14] or desamidation processes [10].

Only the EE isozyme is commercially available. Depending on the manufacturer, the enzyme batches contain varying amounts of impurities above all the isoforms of EE-HLADH and the ES isozyme. Several preparation methods for the ES and SS isozyme have been published (e.g. Refs. [8] and [15]–[18]). In the following we will describe a "large-scale" preparation method for the three main isozymes and their prime fractions which avoids high-pH steps in the separation of the isoforms from the main isozymes.

The preparations of the SS isozyme and the EE isoform include an AMP-Sepharose affinity chromatography step. The coenzyme used in the eluent is fast and quantitatively removed in an additional CM chromatography step.

Until now digestion and fingerprinting of the fragments obtained was unable to detect differences between the EE isozyme and its isoform [19]. For the first time we were able to show a mass difference by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) which we could relate to a glycosylation of EE-HLADH. This result corresponds to recent findings of glycosylated HLADH in commercial preparations, where a non-enzymatic glycation of lysine residues was identified [20,21]. Thus the old question concerning the nature of the isoforms in HLADH preparations could be answered.

2. Experimental

2.1. Materials and general procedures

CM-Sepharose CL6B and 5′-AMP-Sepharose were obtained from Pharmacia (Uppsala, Sweden) and DEAE-23-SH-Cellulose from Serva (Heidelberg, Germany). All materials were pretreated according to the instructions of the manufacturers and packed in glass columns. The flow-rate was regulated by a peristaltic pump and the elution profile was recorded with a flow detector at 280 or 254 nm, respectively (all chromatography equipment from LKB (Bromma, Sweden). Buffer salts (p.a. quality) and electrophoresis chemicals were purchased from Serva. The pH values given were measured and adjusted at room temperature.

The final yields of purified homogeneous enzyme species were determined spectroscopically using an absorption coefficient of 0.455 mg^{-1} cm^2 at 280 nm [22]. In the course of enzyme preparations HLADH-containing fractions were identified and concentrations estimated using activity tests. The NADH produced upon oxidation of ethanol under substrate saturation was followed at 340 nm. In 0.1 *M* N-[Tris–(hydroxymethyl)methyl]-3-aminopropanesulfonic acid (TAPS) pH 8.5, 0.5 m*M* NAD (Boehringer, Mannheim, Germany), 10 m*M* ethanol the maximal turnover rates are 3.8 s^{-1} for the EE isozyme, 2.2 s^{-1} for the ES isozyme and 1.1 s^{-1} for the SS isozyme (for experimental details see Ref. [23]). The steady-state activities obtained were used to calculate the enzyme concentrations.

2.2. Electrophoresis

The purity of the enzyme species from other proteins was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the following conditions. The gel composition was 12% T–2.7% C (0.75 mm thick, size: 8 × 6 cm). As gel buffer 0.375 *M* Tris–HCl pH 8.8 with 0.1% SDS (w/v) was used. For the stacking gel (4% T) the buffer was 0.125 *M* Tris–HCl pH 6.8 with 0.1% SDS. The electrode buffer was 0.19 *M* glycine–0.025 *M* Tris–0.01% SDS pH 8.3. Gels were run at 150 V (not exceeding 30 mA current).

The homogeneity of isozymes and isoforms was investigated by native electrophoresis on polyacrylamide gels (3.75% T, 2.7% C, 0.5 mm thick). As gel buffer 50 m*M* Tris–acetate pH 7.5 was used. Gels were polymerized overnight (40 μl of 10% ammonium persulfate/20 ml) and run in 50 m*M* Tris–acetate pH 6.0 at 150 V (not exceeding 25 mA) for up to 3 h. Protein was visualized with Coomassie brilliant blue or with

an activity staining using nitrotetrazolium blue, phenazine methosulfate, ethanol and NAD [17].

Isoelectric focusing was done on 100-μm polyacrylamide gels (14 × 8 cm) with pH gradients from 4 to 9 (Ampholytes from Serva; after prefocusing (30 min, 400 V) the protein focusing was carried out for 30 min at 400 V and 2–3 h at 800 V.

2.3. Characterization of proteins

Zinc contents were determined by atomic absorption spectroscopy. Free sulfhydryl groups were determined according to Ellmann [24]. Enzyme concentrations of 8–12 μM in 0.1 M Tris pH 8.0, 1 mM EDTA, 1% SDS were used. The concentration of the reagent 5,5′-dithiobis-2-nitrobenzoate (Fluka, Buchs, Switzerland) was 0.3 mM. The absorption was measured in a Lambda 9 spectrophotometer (Perkin-Elmer, Überlingen, Germany) against a blank without enzyme at 412 nm and the concentration was calculated with a absorption coefficient of 13 600 M^{-1} cm^{-1} of the resulting product.

Glycosylation of the protein was shown by a periodate oxidation-based release of formaldehyde, which was determined fluorimetrically as a derivative obtained from a subsequent reaction with acetylacetone and ammonia [25]. To remove interfering phosphate the protein was dialyzed against 80 mM sodium sulfate. Quantitative determinations were performed with 5 and 20 nmol of alcohol dehydrogenase, respectively.

Determinations of molecular masses were done on a time-of-flight mass spectrometer with laser desorption (MALDI-MS, Vision 2000, Finnigan, Bremen, Germany). The protein (2 mg ml^{-1}) was measured in 20 mM Tris–HCl pH 7.5.

3. Results

3.1. Preparation of horse liver alcohol dehydrogenase isozymes and isoforms

All steps of the preparation were carried out at 4°C or on ice. Fresh horse liver (5 kg; freed from big vessels, connective tissue and fat) was homogenized in a meat grinder and extracted with 3 l of 50 mM K-phosphate buffer pH 7.4 for 90 min under stirring. After centrifugation (14 000 g, 45 min; a Sorvall Superspeed RC 2-B, Du Pont, USA, equipped with a GS3 rotor was used for all centrifugation steps), the supernatant was subjected to a heat denaturation step. Under stirring the solution was rapidly heated in a water bath (70–80°C) up to 50°C and kept for 15 min at this temperature and subsequently cooled again to 4°C (on ice–NaCl). The turpid solution obtained was undergone a first ammonium sulfate precipitation. During 60 min, finest pulverized ammonium sulfate was slowly added under stirring to give 40% saturation (226 g/l). After another 60 min of stirring the resulting precipitate was removed by centrifugation (14 000 g, 1 h). To the clear red supernatant that contained the enzymatic activity, additional ammonium sulfate was added to give 70% saturation (180 g/l) during 1 h. This precipitation step could be performed overnight under continuous stirring. After centrifugation (14 000 g, 1 h), the enzyme-containing precipitate was resuspended in the lowest possible volume (usually 300 ml) of 10 mM Tris–HCl pH 8.2 and dialyzed against 10 l of the same buffer for two days with four changes. The next step was DEAE chromatography on a Buchner funnel (15 cm diameter; 2.5 l of ion-exchange material) equilibrated with 10 mM Tris–HCl pH 8.2 which was also used as the eluent. Under these conditions almost no enzymatic activity is retained, whereas, e.g., hemoglobin is bound to the resin. Flow-rates were adjusted with a moderate vacuum. The ethanol-active fractions of the slight yellow eluate were collected and dialyzed against 5 l of 5 mM Na-phosphate buffer pH 7.0 (two additional changes in intervals of 12 h).

The HLADH isozyme mixture was loaded on a CM-Sepharose ion-exchange column (30 cm × 7 cm; flow-rate 400 ml h^{-1}) equilibrated with 5 mM Na-phosphate pH 7.0. Non-binding proteins were removed by extensive rinsing with the equilibration buffer. HLADH was fractionated with a three-step gradient in ionic strength; 15 mM Na-phosphate was used to elute the EE isozyme in a large fraction with a preceding lower peak containing mainly the EE isoform.

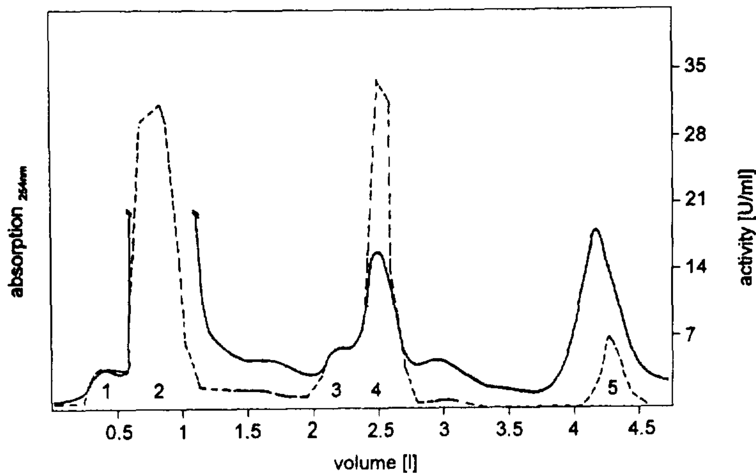

Fig. 1. Separation of HLADH isozymes on CM-Sepharose: elution diagram of the CM-Sepharose column (20 × 7 cm I.D.) used for the separation of the isozymes and isoforms of horse liver alcohol dehydrogenase. Solid line, absorption at 254 nm; dashed line, activity with 5.7 m*M* ethanol, 0.5 m*M* NAD in 0.1 *M* glycine pH 10 (determined photometrically at 340 nm). The column was equilibrated and washed (after protein loading) with 5 m*M* phosphate buffer pH 7.0; for elution 15 m*M* phosphate was used and the ionic strength stepwise increased to 20 m*M* (after 1.75 l elution volume) and 25 m*M* (after a 3.5-l elution volume). 1, Isoform of the EE-HLADH; 2, EE-HLADH isozyme; 3, isoform of ES-HLADH; 4, ES-HLADH isozyme; 5, SS-HLADH isozyme.

The ES isozyme and its isoform were eluted with 20 m*M*, the SS isozyme with 25 m*M* Na-phosphate pH 7.0, respectively (Fig. 1). The SS isozyme was eluted together with an ethanol-inactive protein fraction.

At this stage of the preparation the different isozymes and isoforms are separated and purification to homogeneity is different for all of them. The purity of the EE isozyme is already high enough to allow crystallization as the final purification step. After addition of 100 m*M* Na-sulfate to avoid amorphous precipitation, the EE-HLADH-containing fractions were combined (usually 2 mg ml^{-1}) and concentrated to give 15–20 mg ml^{-1} by ultrafiltration (Amicon ultrafiltration cell with PM30 membrane, Amicon, Witten, Germany). After centrifugation to remove the precipitated material the clear supernatant was packed in dialysis tubings and the enzyme was crystallized from 30 m*M* Tris–HCl pH 8.4 by stepwise addition of *tert.*-butanol (p.a. grade) to a final concentration of 18% (v/v) during two weeks. After centrifugation the crystals can be dissolved in 150 m*M* Na-sulfate. The purity is >98% with maximum activity and two zinc ions per subunit. To remove residual traces of isoforms a re-crystallization results in usually 1.5–2.5 g of EE isozyme where no protein impurities can be detected by any means. The enzyme can be stored in liquid nitrogen for many years without loss of activity as a frozen solution (20–90 mg ml^{-1} in 150 m*M* Na-sulfate).

The ES isozyme usually can be crystallized from 30 m*M* K-phosphate pH 8.8 with *tert.*-butanol with a final concentration of 25% (v/v). The applicability of this preparation step depends on the amount of impurities still present in the ES-HLADH fractions. To obtain high yields a re-chromatography on CM-Sepharose using a linear gradient from 15 to 20 m*M* Na-phosphate pH 7.0 or an affinity chromatography step as described below for the SS isozyme is necessary. The yields obtained range from 300 to 800 mg.

The SS isozyme was further purified in an affinity chromatography step on 5′-AMP-Sepharose. The pooled fractions were loaded to a column (5 cm × 2 cm, 20–26 ml h^{-1} flow-rate) equilibrated with 50 m*M* Na-phosphate pH 7.5. Non-binding proteins were eluted with the same buffer. The SS isozyme was eluted by addition of

0.2 m*M* NAD and 1.5 m*M* cholic acid to this buffer. In this step a strong ternary complex, SS-HLADH–NAD–cholic acid, is formed in concurrence to the coenzyme-analogous 5′-AMP. The enzyme is immediately eluted in concentrations >5 mg ml^{-1}. Due to the high stability constant of the ternary complex obtained it is almost impossible to remove coenzyme and inhibitor quantitatively by dialysis. Therefore we introduced an additional ion-exchange step. After dialysis against 5 m*M* Na-phosphate pH 7.0 the enzyme was loaded on a CM-Sepharose column (20 cm × 2 cm; flow-rate 60 ml h^{-1}) equilibrated with the same buffer. The column was extensively washed with the same buffer until no further decrease of the absorption at 254 nm could be detected (usually 10–12 h). The pure SS isozyme could be eluted with 25 m*M* Na-phosphate pH 7.0–125 m*M* Na-sulfate. The enzyme concentration of the fractions obtained is 5–15 mg ml^{-1} and the highest concentrated fractions can be immediately frozen in liquid nitrogen where the enzyme can be stored. The lower concentrated fractions were further concentrated by ultrafiltration. Typical yields for the SS isozyme are 20–400 mg depending on the liver used (see Discussion).

To remove non-ADH proteins from the isoform of the EE-HLADH (first peak in the elution diagram in Fig. 1), this enzyme fraction was also loaded to the AMP affinity gel and washed with 50 m*M* Na-phosphate pH 7.5. In contrast to the SS isozyme, it was necessary to use higher concentrations of coenzyme for elution: 4.5 m*M* NAD and 3 m*M* cholic acid in the same buffer. The resulting fraction still contains the EE isozyme and its isoform. The separation of the isoform from contaminating EE isozyme was done subsequently by an ion-exchange chromatographic step with gradient elution. The AMP eluate had to be extensively dialyzed against 5 m*M* phosphate buffer pH 7 because a low ionic strength is necessary for binding of the EE isoform to the CM material (column 20 cm × 2 cm; flow-rate 48 ml h^{-1}). The column-bound protein was extensively washed with 5 m*M* Na-phosphate pH 7.0 to remove residual NAD (Fig. 2). The separation was achieved by gradient

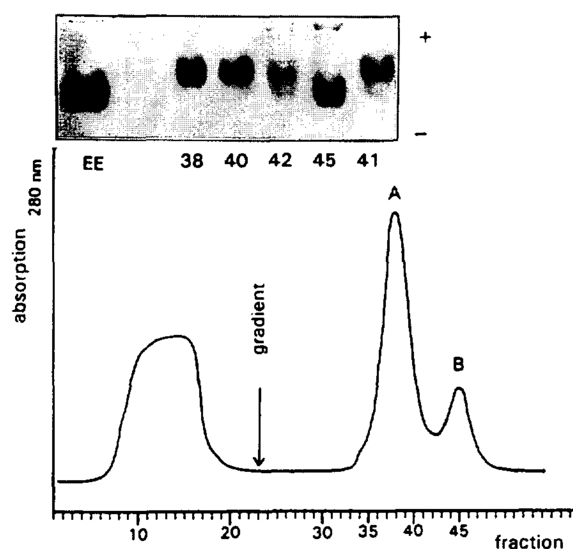

Fig. 2. Separation of EE-HLADH and EE-HLADH isoforms. The chromatography was done on a CM-Sepharose column (20 × 2 cm I.D.) equilibrated with 5 m*M* phosphate buffer pH 7.0 (flow-rate 48 ml/h); for elution a linear gradient from 10 to 25 m*M* phosphate was used (125 ml each) and 8-ml fractions were collected; peak A contains the EE-HLADH isoform, peak B the EE-HLADH. Inset: gel of a native PAGE of HLADH fractions showing the complete separation of the EE isoform and the EE isozyme in the elution peaks A and B.

elution with increasing ionic strength (10 m*M* to 25 m*M* phosphate pH 7.0, 2 × 125 ml). The yield of EE isoform is 20–100 mg. It is only stable in concentrations of 2–8 mg ml^{-1} and can be stored in liquid nitrogen.

3.2. Characterization of the EE-HLADH isoform

Analytical isoelectric focusing resulted in isoelectric points of 7.8 for the EE isoform and 8.15 for the EE isozyme (Fig. 3). By atomic absorption spectroscopy 1.97 (±0.04) zinc per subunit were found. Identical values are found for the EE and SS isozyme.

The migration behaviour on native polyacrylamide gels was unaffected by treatment with dithiothreitol or β-mercaptoethanol (up to 1400 times molar excess). With the Ellman reaction, the amount of free sulfhydryl groups (14 ± 0.2

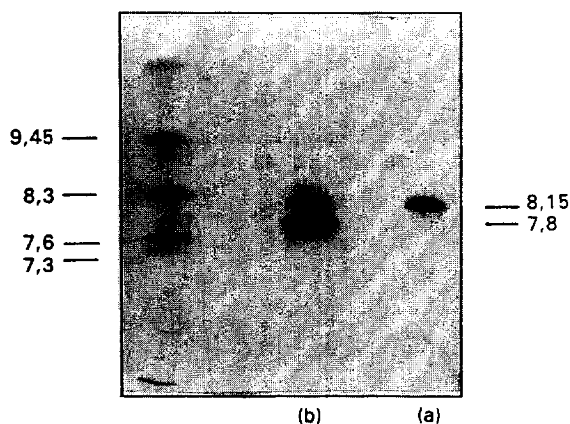

Fig. 3. Isoelectric focusing of EE-HLADH (a) and its isoform (b). Conditions are described in the Experimental section. The p*I* standards used on the left line are 9.45 for ribonuclease, 8.3/7.6 for myoglobin from whale and 7.3 for myoglobin from horse. In this representation some EE-HLADH is still left in the isoform fraction. Residual impurities of EE-HLADH could be removed by re-chromatography on CM-Sepharose.

per subunit) was the same for the EE isozyme and its isoform.

MALDI-MS resulted in a molecular mass of the isoform which is 130–165 relative molecular mass higher as compared to the main EE isozyme (Fig. 4). A comparison of EE-HLADH, SS-HLADH and a mixture of EE and its isoform proved the reliability of the mass difference obtained. Thus, it could be concluded that these two fractions are chemically different. Masses in Fig. 4 are related to single subunits without zinc. The two subunits are separated and the alcohol dehydrogenase loses its zinc at pH lower than 5 as is the case in the matrix for the MALDI-MS experiments. Although the method shows a tolerance of 0.1%, the resulting mass for the SS isozyme of 39511.9 is almost identical with the value of 39510.7 obtained from the amino acid sequence of the protein.

For commercially available HLADH it has been reported that a part of the enzyme is glycosylated. A glycation of Lys-231 at the surface of the molecule as the product of non-enzymatic glycation has been shown [20,21].

The method of Gallop et al. [25] was applied to all isozymes and the EE-HLADH isoform, but only with the isoform sugar could be determined. We found 0.4 mol sugar/mol of enzyme. The existence of a glycosylated residue is in accordance with the higher molecular mass obtained. The p*I* is lowered in the isoform. From the EE isozyme we produced an artificial glycosylation product according to Ref. [26] which shows identical electrophoretic behaviour. The decrease in p*I* corresponds to the disappearance of one positive charge per monomer. Since no heterogeneity of the isoform could be detected by MALDI-MS or by monomerization and re-hybridization experiments with subsequent native PAGE (data not shown) we conclude that the low amount of 0.4 mol sugar/mol of enzyme detected corresponds to a non-quantitative determination in the analytical procedure.

Using MS and Edman degradation we were able to show that no differences between EE-HLADH and its isoform exist in the N- and C-terminal regions of the proteins. In a cyanogen bromide digest of the EE isoform the N-terminal peptide has the identical mass as compared to the EE isozyme. The C-terminal peptide shows the identical length and sequence as the main form.

Taking all evidence together led us to the conclusion that the EE isoform is the same glycosylation product as described in Refs. [20] and [21].

4. Discussion

The purification procedure presented uses different techniques which have been described earlier for preparations of HLADH. The combination and optimization resulted now in a method which allows the preparation of large quantities of pure HLADH isozymes and isoforms in parallel. Typical yields that can be obtained within three weeks are 1.5–2.5 g of EE-HLADH, 300-800 mg of ES-HLADH, 20–400 mg of SS-HLADH and 50–100 mg of EE-HLADH isoforms from 5 kg of horse liver. Additional isoforms of the ES and SS isozymes are found in some preparations, depending on

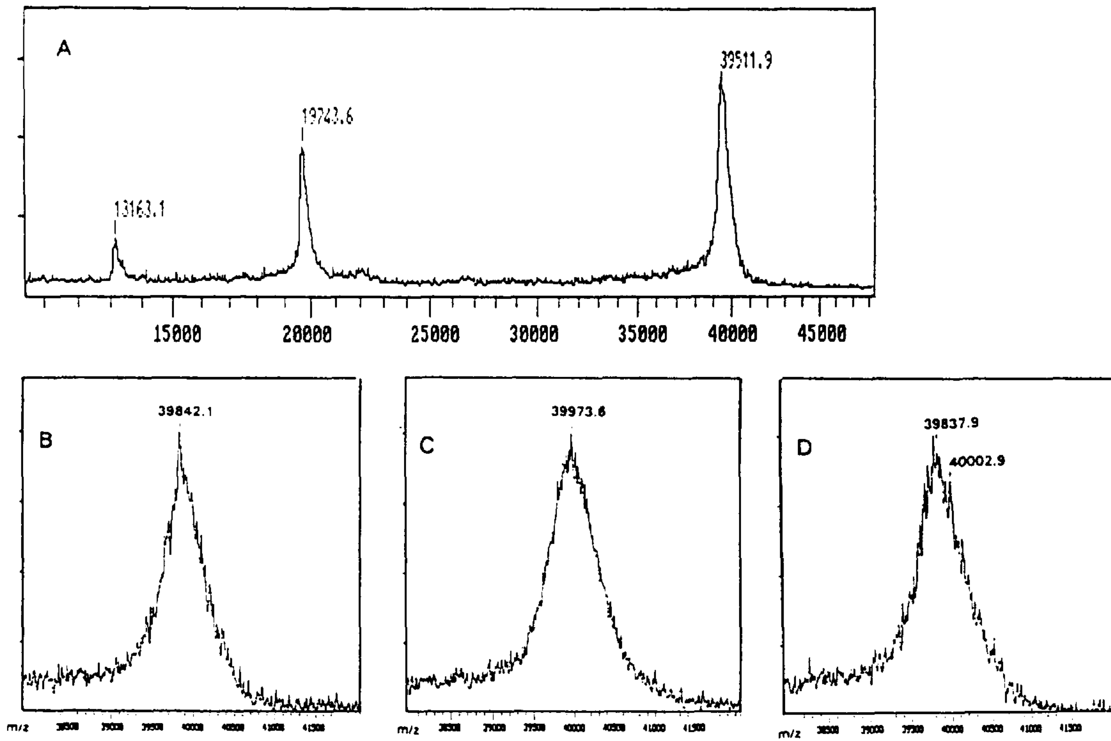

Fig. 4. MALDI-MS of the SS isozyme (A), the EE isozyme (B), the EE isoform (C) and a 1:1 mixture of both (D); the matrix for the laser desorption was 2,5-dihydroxybenzoic acid (10 mg/ml), which was mixed in a 1:1 relation with the protein (2 mg/ml). Masses (theoretical values in brackets) obtained are: 39 511.9 (39 510.7) relative molecular mass for SS-HLADH, 39 842.1 (39 822.7) relative molecular mass for EE-HLADH and 39 973.6 relative molecular mass for the EE-HLADH isoform alone or 40 002.9 relative molecular mass in a 1:1 mixture of EE-HLADH and its isoform. A mass determination of the heterodimeric ES-HLADH resulted in 39 828 relative molecular mass for the E subunit and 39 566 relative molecular mass for the S subunit (data not shown).

the liver used. After many preparations we found that the total amount of HLADH as well as the isozyme and isoform composition varies in a wide range. The SS isozyme is sometimes found in only small quantities or not at all. Until now we were not able to find criteria which allow a prediction of the yields to be obtained (sex, race, age of horses). We found that older horses show higher total contents of HLADH and larger fractions of the isoforms throughout. The occurrence of isoforms was observed in many proteins [27] and is considered to be an aging process of the proteins.

EE-HLADH is a commercially available enzyme which is widely used for synthetic and analytic purposes. The enzyme catalyzes the regio- and stereoselective oxidation of alcoholic groups and the reduction of aldehydes and ketones. HLADH has a very broad spectrum of substrates accepted (several hundreds of compounds have been described in the literature; for a review see Ref. [28]). Usually the commercially available enzyme is used without further purification. The substrate specificity and the stereoselectivity of the HLADH isozymes, however, are significantly different as well as the inhibition patterns (to be published elsewhere), thus reliable data and specific syntheses require the use of pure isozymes. We found that commercially available EE-HLADH usually contains varying amounts of impurities, namely ES-HLADH and isoforms of EE- and ES-HLADH. Thus some activity of the S subunit is always present. A part of the methods described here

allows a very easy purification of the EE isozyme to homogeneity from commercial samples. The SS isozyme additionally accepts steroids as substrates and may also be of interest for organic syntheses. Most preparations described earlier are more difficult, time-consuming, and resulted in very low yields.

The isoforms show only minor differences in the catalytic behaviour but strongly modified stabilities (to be published elsewhere). With the procedure described we were, for the first time, able to prepare 100-mg quantities of an EE-HLADH isoform to homogeneity. The characterization and the comparison with literature data about a glycosylated fraction of commercially available HLADH [20,21] led us to the conclusion that the EE-HLADH isoform described here is glycated at one Lys residue. The disappearance of one positive charge per subunit, however, is sufficient to allow a complete separation using conventional techniques in a preparative scale.

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