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Purification and Characterization of 2-Alkenal Reductase*

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Summary: The purification of a 2-alkenal reductase to homogeneity from a rat liver 100000 × *g* supernatant is described. Its molecular weight has been determined by Sephadex G-100 chromatography and sodium dodecylsulfate polyacrylamide gel electrophoresis before and after reduction with mercaptoethanol and carboxymethylation.

The monomeric form has a molecular weight of 45000. It tends to form, to a very small extent, dimeric and trimeric aggregates of molecular weights 90000 and 135000.

The isoelectric point (IP) was determined to be 6.2 by isoelectric focusing.

Reinigung und Charakterisierung von 2-Alkenal-Reduktase

Zusammenfassung: Es wird die Isolierung der 2-Alkenal-Reduktase aus Rattenleber-100000 x*g*-Überstand und ihre Reinigung bis zur Homogenität über $(NH_4)_2SO_4$-Fällung, Sephadex-, DEAE- und CM-Cellulose-Chromatographie mit einer rund 600fachen Anreicherung beschrieben.

Das Molekulargewicht wurde durch Sephadex G-100-Chromatographie und Natriumdodecylsulfat-Polyacrylamidgel-Elektrophorese vor und nach Reduktion mit Mercaptoäthanol und Carboxymethylierung bestimmt. Die monomere Form hat ein Molekulargewicht von 45000. Es bildet zu einem sehr geringen Anteil dimere und trimere Aggregate vom Molekulargewicht 90000 und 135000.

Der isoelektrische Punkt (IP) wurde zu 6.2 durch isoelektrische Fokussierung bestimmt.

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Enzymes:
2-Alkenal reductase, 2-alkenal: NADPH oxidoreductase (EC 1.3.1.? ; not yet listed)
Sphingosine* kinase, ATP: sphingosine 1-phosphotransferase (EC 2.7.1.? ; not yet listed)
Sphingosine-1-phosphate aldolase (lyase), sphingosine-1-phosphate alkanal lyase (EC 4.1.2.? ; not yet listed).

* *Note:* Sphingosine is a generic name for all naturally occurring long-chain bases.

The degradation of the predominant long-chain base 4*t*-sphingenine is initiated by the ATP-dependent phosphorylation of the primary alcohol group of D-*erythro*-amino-1,3-dihydroxyoctadec-4*t*-ene. The phosphate ester is then cleaved in a pyridoxal phosphate-catalyzed lyase reaction to phosphorylethanolamine and 2*t*-hexadecenal. Since the C-3 to C-18 fragment of 4*t*-sphingenine labelled in the hydrocarbon chain was recovered as labelled palmitic acid from ester and amide lipids of rat liver after intravenous administration, the question of the immediate pathway of 2*t*-hexadecenal was raised. Neither in experiments in vivo nor in vitro were we able to find 2-*trans*-hexadecenoic acid, but we detected a 2-alkenal reductase in the cytosol of rat liver, which stereospecifically and chain-length specifically transfers the *pro R* (A-side) NADP^{2}H to C-3 of the substrate 2*t*-hexadecenal, yielding palmitaldehyde. Palmitaldehyde may then be either oxidized to palmitic acid or reduced to hexadecanol and further utilized as described before[1].

The oxidation of the reduced pyridine nucleotide in the reduction reaction of 2*t*-hexadecenal facilitated a simple enzyme assay, and in turn made attempts at the purification of the 2-alkenal reductase feasible[2,3].

In this communication, we describe the purification to homogeneity of 2*t*-alkenal reductase from the rat liver 100000 × *g* supernatant and, in addition, properties of the purified enzyme.

Experimental

Substrates: ^{3}H-labelled 2-*trans*-hexadecenal (specif. radioactiv. 1.9 mCi/mmol) was synthesized in this laboratory[2].

The *optical assay* and *radio thin-layer analysis* have been described in the preceeding paper[3].

Enzyme purification: All purification steps were carried out at 4 °C in the cold room.

Six livers from 250 - 300-g rats were homogenized in 0.1M Tris/HCl buffer, pH 6.8, and the 100000 × *g* supernatant fraction was obtained by *ultracentrifugation*[4]. Alcohol dehydrogenase was inactivated by dialysis of the 100000 × *g* supernatant against 100 vol. of 0.05M Tris maleate buffer, pH 5.7, for 72 h. The buffer was changed after 24 and 48 h[5]. Precipitated protein was centrifuged at 12000 × *g* for 60 min.

$(NH_4)_2SO_4$ precipitation: The pH of the 100000 × *g* supernatant was then adjusted to pH 6.4 with concentrated ammonia. Solid $(NH_4)_2SO_4$ was added to 50% saturation. The precipitate which formed within 2.5 h was sedimented by centrifugation at 12000 × *g* for 60 min, and the supernatant saturated to 80% $(NH_4)_2SO_4$. The protein which precipitated within 2.5 h was pelleted by centrifugation (12000 × *g*, 60 min), the pellet was dissolved in 10 - 15 m*l* 0.1M Tris-HCl buffer, pH 6.8, and the solution was dialyzed against 2 *l* of the same buffer.

Sephadex G-200 filtration: The dialyzed enzyme solution is passed over a Sephadex G-200 column (2.6 × 95 cm) equilibrated with 0.1M Tris/HCl buffer, pH 6.8. 4.0-m*l* fractions were collected and assayed for enzyme activity. The enzyme-containing fractions were pooled and concentrated by ultrafiltration using an UM2 Diaflo ultrafiltration membrane.

DEAE-Cellulose chromatography: The concentrated enzyme solution was dialyzed against 0.01M Tris/HCl buffer, pH 8.2, and fractionated on a DEAE cellulose column (2.6 × 25 cm), Whatman DE-52, microgranular. The loaded column was washed with 60 m*l* buffer. Then a linear gradient made by adding 400 m*l* 0.25M NaCl in 0.01M Tris/HCl buffer, pH 8.2, to 400 m*l* of the some buffer was started. Again 4.0 m*l* fractions were collected and the enzyme activity of the fractions determined by the optical assay. The pooled enzyme fractions were concentrated by ultrafiltration.

CM-cellulose chromatography: The concentrated protein solution (4.0 m*l*) was dialyzed against 2 × 1000 m*l* 0.01M phosphate buffer, pH 5.6 and then loaded on a CM-cellulose column (2.6 × 18 cm) equilibrated with the same buffer. The column was washed with 50 m*l* of the buffer before starting a linear gradient made by adding 250 m*l* 0.2M NaCl in the phosphate buffer to 250 m*l* of the same buffer. 2.0-m*l* fractions were collected. The enzymatically active fractions were pooled and again concentrated by ultrafiltration.

Disc electrophoresis was carried out in 7% acrylamide separating gels with 2.5% acrylamide stacking gel at pH 8.9.

The molecular weight of the reductase was determined by sodium dodecylsulfate gel electrophoresis in 7.5% acrylamide gels according to Weber and Osborn[6]. The protein samples were heated for 3 min at 95 °C in the presence of 1% sodium dodecylsulfate, 1% mercaptoethanol and 4M urea. After cooling to 4 °C, the protein was carboxymethylated with iodoacetamide according to established procedures[7,8]. Myoglobin (17200 daltons), ovalbumin (45000 daltons), human serum albumin (68000 daltons) and phosphorylase a (94000 daltons) served as reference proteins. The electrophoresis

was carried out in 0.1M phosphate buffer pH 7.2, 0.1% in sodium dodecylsulfate, at 3.0 mA/gel.

Isoelectric focusing was carried out according to Vesterberg[9] with 1.4 mg enzyme protein in a 110-m*l* column with a linear pH gradient between pH 3 and 10 in a linear sucrose gradient (0 - 40%). Initial voltage 400 V; after 8 h, 800 V for 44 h. Temperature 4 °C. 3-m*l* fractions were collected at the end of the experiment and tested for enzymic activity after adjusting the pH to 6.8.

The molecular weight of the alkenal reductase was determined by filtration on a Sephadex G-100 column[10] (1.6 × 85 cm). 2.0-m*l* fractions were collected and the enzyme activity measured as described before. For calibration, the elution volumes of aldolase (147000), human serum albumin (68000), ovalbumin (45000), chymotrypsin (25000) and myoglobin (17200) were determined, and the void volume of the column was measured with dextran blue 2000.

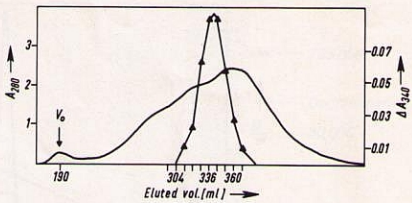

Fig. 1. Sephadex G-200 filtration of dialyzed $(NH_4)_2SO_4$ fraction (50 - 80% saturation).
315 mg protein (1.73 U) dissolved in 10 m*l* 0.1M Tris buffer pH 6.8 was applied to the column (2.6 × 95 cm); flow rate: 18 ml/h, 4-m*l* fractions were collected.
—— extinction at 280 nm; —▲— enzyme activity (ΔA_{340} nm/5min).

Results

One of the non-membrane bound enzymes of sphingosine metabolism in the mammalian cell is the 2-alkenal reductase present in different organs. Its function is the reduction of 2*t*-hexadecenal to palmitaldehyde, a reaction which immediately follows the cleavage of the 1-phosphate ester of the predominant long-chain base, 4*t*-sphingenine.

We localized this enzyme in the cytosol (100000 × *g* supernatant), described the absolute requirement for NADPH, the side-specific hydrogen transfer from the A side (*pro R*) of NADPH, its chain length specificity for α,β-unsaturated aldehydes with 14 to 16 carbon atoms, and the organ distribution of the 2-alkenal reductase[3].

The solubility of the enzyme made an attempt at purification feasible.

The first step of the enzyme enrichment consisted in the cell fractionation of rat liver. The 100000 × *g* supernatant was dialyzed against Tris/maleate buffer, pH 5.6, for 72 h[5] in order to inactivate the alcohol dehydrogenase and thus allow the combined optical test. The dialyzed fraction was subjected to ammonium sulfate precipitation and the protein fraction precipitating between 50-80% saturation was chromatographed on Sephadex G-200 (Fig. 1). The enzyme-containing fractions of the eluate were concentrated by ultrafiltration and chromatographed on DEAE cellulose at pH 8.2, applying a linear sodium chloride gradient between 0 and 0.25M, Fig. 2. This step led to a 10 to 15-fold enrichment of the 2-alkenal reductase. Finally, CM-cellulose chromatography, Fig. 3, at pH 5.6 with a 0 to 0.25M NaCl gradient yielded a homogenous protein fraction, as demonstrated by polyacrylamide gel electrophoresis. Fig. 4 a-c displays the results of the purification steps by the three chromatographic procedures as made visible by sodium dodecylsulfate gel electrophoresis. The Table summarizes the details of the purification. The isoelectric point of the reductase was determined to be 6.2 by isoelectric focusing using an ampholine gradient between pH 3 and 10[9] in a 110-m*l* LKB column, Fig. 5. The molecular weight was determined to be 45000 by sodium dodecylsulfate-polyacrylamide gel electrophoresis, and about 52000 by Sephadex G-100 filtration after $(NH_4)_2SO_4$ precipitation in conjunction with calibrating proteins, Fig. 6 A, B.

In order to find out whether the enzyme occurs as a multimeric enzyme, and if so, to determine the molecular weight of the smallest subunit, the enzyme was treated under denaturing and reducing conditions (1% sodium dodecylsulfate, 4M urea, 3 min at 95 °C, 1% mercaptoethanol[7,8]) and subsequently carboxymethylated with iodoacetamide. The results of the Sephadex G-100 filtration and sodium dodecylsulfate-polyacrylamide gel electrophoresis were in good agreement. The homogenous protein had a molecular weight of 45000.

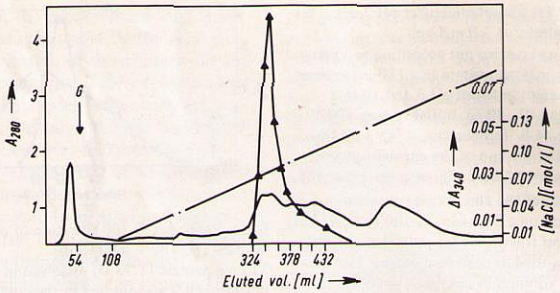

Fig. 2. DEAE-cellulose chromatography of the combined enzyme fractions from Sephadex G-200 filtration.
138 mg protein (2.07 U) was dialyzed against 0.01M Tris buffer, pH 8.2 and separated on a 2.6 × 25 cm DEAE-cellulose column by applying a linear gradient made from 400 ml buffer and 400 ml 0.25M NaCl in the same buffer. Flow rate: 20 ml/h; 4-ml fractions.
— extinction at 280 nm; —▲— rel. enzyme activity ($\Delta A_{340\ nm}$/5 min); –·– salt gradient concentration.

Table. Purification of the 2*t*-alkenal reductase from rat liver 100000*xg* supernatant.

Procedure	Protein [mg]	Spec. activ. [mU/mg]	Total activ. [mU]	Yield [%]	Purification factor
72 h dialysate	1410	2.5	3520	100	1.0
$(NH_4)_2SO_4$ (50 - 80% saturation)	630	5.5	3460	98	2.2
Sephadex G-200 filtration	138	15	3460	59	6.0
DEAE-cellulose chromatography	6.2	155	960	27	62
CM-cellulose chromatography	0.17	1570	260	7.5	630

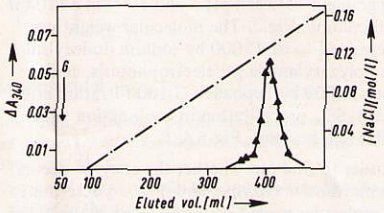

Fig. 3. CM-cellulose chromatography of 6.2 mg enzyme protein (0.96 U) pooled from the DEAE-cellulose chromatography.
Elution buffer: 0.01M potassium phosphate buffer, pH 5.6, with a gradient between 250 ml buffer and 250 ml 0.2M NaCl in the same buffer. Column dimensions: 2.6 × 18 cm; flow rate: 20 ml/h.
—▲— Relative enzyme activity; –·–·–·– salt gradient concentration.

Reduction of the native enzyme with mercaptoethanol and subsequent sodium dodecylsulfate electrophoresis revealed mainly the 45000-dalton protein. A weak band appeared at 90000 daltons, Fig. 6A, b. If no reductive treatment of the enzyme occurs again, the 45000 monomer is the predominant component, and additional faint bands with 90000 (dimer), 135000 and even higher molecular weights are visible in the gel. We interpret these results as follows: The enzyme has a molecular weight of 45000. The ionic strength of the eluting buffer (0.1M) apparently is not able to prevent completely a reversible aggregation. When the protein is fully carboxymethylated, no aggregation is observed. The reduction with mercaptoethanol followed by carboxymethylation under denaturating conditions apparently exposes SH-groups not readily accessible to sulfhydryl reagents, since increasing concentrations of iodoace-

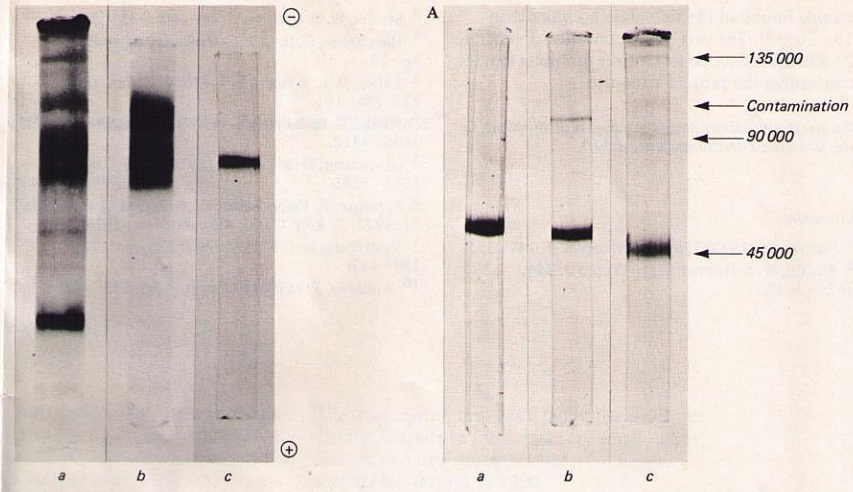

Fig. 4. Polyacrylamide gel electrophoresis of enzymatically active protein fractions at different stages of the purification.
a) After Sephadex G-200 chromatography, b) after DEAE-cellulose chromatography, c) after CM-cellulose chromatography. The conditions of the electrophoresis are reported under Experimental.

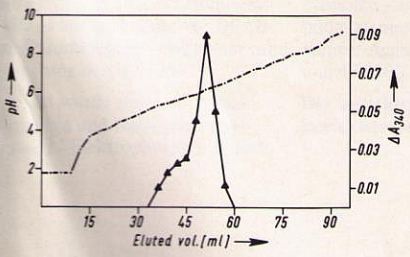

Fig. 5. Isoelectric focusing of pooled and concentrated enzyme fractions of DEAE-cellulose chromatography (1.4 mg; 0.375 U) in a 110-ml LKB-column.
A linear pH gradient between pH 3 and 10 was built up with 1% ampholine in a linear sucrose gradient (0 - 40 %). Initial voltage: 400 V, after 8 h, 800 V for 44 h.
Temperature 4 °C. –·–·– pH gradient; —▲— relative enzyme activity (ΔA_{340nm}/5 min).

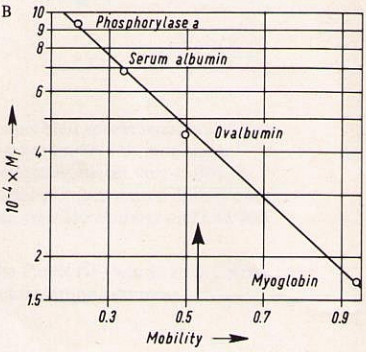

Fig. 6. A) Sodium dodecylsulfate-polyacrylamide gel electrophoresis of alkenal reductase.
a) Enzyme protein after reduction and carboxymethylation, b) after reduction with 0.1% mercaptoethanol, c) without reduction.
B) Molecular weight determination of alkenal reductase by sodium dodecylsulfate-polyacrylamide gel electrophoresis.
Abscissa: electrophoretic mobility of reference proteins; ordinate: logarithms of molecular weights of reference proteins.

tamide inhibited the reductase no more than 15 - 20%[3]. The very small concentration of the 2*t*-alkenal reductase in rat liver prohibits studies concerning the protein structure.

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