Co-purification of human serum lipoamidase and biotinidase: evidence that the two enzyme activities are due to the same enzyme protein

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A more than 20000-fold purification of human serum lipoamidase is described. This was accomplished by $(\text{NH}_4)_2\text{SO}_4$ precipitation and chromatography on DEAE-Sephacel, Blue Sepharose CL-6B and phenyl-Sepharose CL-4B, followed by preparative isoelectric focusing (IEF) and finally by gel-permeation chromatography. Co-precipitation and co-chromatography of lipoamidase and biotinidase activities with equal yields and purification were obtained in all the purification steps, indicating that lipoamidase and biotinidase activities in human serum are due to the same enzyme protein. After preparative

IEF, two fractions with both lipoamidase activity and biotinidase activity were found at pI 4.0 and pI 4.4 respectively. The molecular mass of the enzyme was found to be 76 kDa. When 2-mercaptoethanol was used instead of cysteine as stabilizer during the purification procedure, only one major form (pI 4.0) of the enzyme was obtained after preparative IEF. By addition of cysteine, this form was transformed to an enzyme with pI 4.4, indicating that this latter form is a cysteine adduct, produced during the IEF procedure.

INTRODUCTION

Lipoic acid (6,8-dithio-octanoic acid) is a coenzyme of 2-oxoacid dehydrogenases and is covalently bound to the ϵ -amino groups of lysine residues in these enzymes [1,2]. About 30 years ago a bacterial lipoamidase (named lipoyl-X-hydrolase) from *Streptococcus faecalis* was described [1,3,4], which hydrolyses lipoyl-lysine and other lipoyl-containing amides. A similar enzyme was described in baker's yeast [5], and later in rabbit and rat tissues [6,7]. The enzyme was identified in human serum [8–10] and breast milk [11], by the use of lipoyl-*p*-aminobenzoic acid (lipoyl-PABA) as an artificial substrate, and the enzyme from breast milk was shown to have a serine residue in the active site [11]. Lipoamidase activity was also found in guinea-pig liver [12] and brain [13], by using lipoyl-PABA as substrate, and was shown to be an integral membrane glycoprotein mainly confined to the microsomal fraction [12].

Recently we compared the lipoamidase activities in human serum and milk by using both lipoyl-lysine and lipoyl-PABA as substrates [14]. The enzyme found in breast milk with lipoyl-PABA as substrate could not utilize lipoyl-lysine as substrate, indicating that lipoamidase in human serum and that in breast milk are different enzymes [14]. The serum and breast milk enzymes showed different inhibition patterns, e.g. serum lipoamidase was strongly inhibited by thiol reagents, whereas milk lipoamidase was unaffected [14].

Lipoamidase has been purified 983-fold [15] from human serum, and it was shown that the enzyme failed to hydrolyse lipoic acid from intact bovine pyruvate dehydrogenase complex. Further, the purified enzyme appeared to have biotinidase activity, but the authors [15] did not discuss the significance of this. Similarly, biotinidase purified from human serum failed to hydrolyse protein-bound biotin [16]. On the other hand, both lipoamidase and biotinidase could hydrolyse lipoyl-lysine and biocytin (biotinyl-lysine) respectively obtained after proteolytic cleavage of lipoyl- and biotinyl-containing proteins and peptides

[15,16]. Garganta and Wolf [17] inactivated more than 95% of both the lipoamidase activity and the biotinidase activity of a partially purified fraction from human serum by use of a monospecific polyclonal antibody against biotinidase, which was obtained after immunization of rabbits with purified biotinidase.

The aim of the present study was to answer the question: Is lipoamidase in serum the same enzyme as biotinidase? We therefore purified the enzyme to homogeneity and determined its activities by using both the natural and the synthetic substrates for the two enzyme functions. Together with the reported purification procedure for the human serum enzyme(s), we show data which indicate that human serum lipoamidase and biotinidase activities are in fact confined to one and the same enzyme protein.

MATERIALS AND METHODS

Chemicals

Biocytin (ϵ -*N*-L-biotinyl-L-lysine) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Cysteamine hydrochloride and 1,2-diacetylbenzene were purchased from Fluka A.G. (Buchs, Switzerland), and α -lipoic acid, *p*-aminobenzoic acid (PABA) and cysteine hydrochloride were from E. Merck (Darmstadt, Germany). DEAE-Sephacel, Blue Sepharose CL-6B, phenyl-Sepharose CL-4B, Pharmalyte 2.5–5 and Sephacryl S-300 HR were purchased from Pharmacia Biosystem Sverige AB (Sollentuna, Sweden). Lipoyl-lysine was prepared as described by Nawa et al. [2]. Biotinyl-PABA and lipoyl-PABA were synthesized by a procedure used for synthesis of *N*-biotinyl-PABA [18].

Assay of enzyme activity

Four different substrates were used for determinations of lipoamidase and biotinidase, e.g. lipoyl-lysine and biocytin, considered to be the natural substrates of the enzymes, and

lipoyl-PABA and biotinyl-PABA, used as artificial substrates. In the present paper lipoyl-PABA was utilized to identify enzyme-containing fractions in chromatographic procedures, and the four substrates were used for quantitative determinations of enzyme activities in pooled fractions and for enzyme characterization after preparative isoelectric focusing (IEF).

The enzyme activities were expressed in arbitrary units, defined as the amount of enzyme which formed 1 nmol of product/min at 37 °C. All assays were performed in duplicate, except in the fractions collected during chromatographic steps.

Lipoamidase assay

Lipoamidase activity was measured both with the natural substrate lipoyl-lysine and with the artificial substrate lipoyl-PABA with some minor modifications of our previously described procedure [14]. When lipoyl-lysine was used as substrate, 0.1 ml of enzyme solution was mixed with 0.5 ml of 0.15 M sodium acetate buffer, pH 4.86, 60 μ l of 0.1 M cysteamine hydrochloride, 30 μ l of 50 mM Na₂EDTA and 0.66 ml of water. After 10 min preincubation at 37 °C the enzyme reaction was started by addition of 0.15 ml of 10 mM lipoyl-lysine dissolved in 0.1 M NH₃, which increases the pH of the assay mixture to 5.5. After 1 h the reaction was terminated by addition of 0.1 ml of 1 M H₂SO₄. To avoid incomplete protein precipitation and to prevent foaming, 50 μ l of 10% (w/v) human serum albumin (HSA) and 25 μ l of 3-methylbutan-1-ol were added immediately after addition of the acid. Liberated lipoate was extracted with 2 ml of hexane/chloroform (7:3, v/v). After centrifugation (2500 g, 10 min) 1.5 ml of the organic phase was re-extracted into 2.0 ml of 0.1 M Tris buffer, pH 8.0, containing 2 mM Na₂EDTA. Lipoate in the aqueous phase (1.5 ml) was then reduced to dihydrolipoate by addition of 0.2 ml of 1.7 M NaBH₄. After 30 min, excess NaBH₄ was destroyed by addition of 1.5 ml of 1.5 M H₂SO₄. Dihydrolipoate was then determined by Ellman reaction by addition of 1 ml of 1 M Tris buffer, pH 7.9, containing 2 mM Na₂EDTA, and 20 μ l of 20 mM 5,5'-dithiobis-(2-nitrobenzoic acid), dissolved in 0.1 M sodium phosphate buffer, pH 7.0. After 5–30 min the A_{412} was determined and the amount of dihydrolipoate formed was obtained from a calibration standard graph.

When lipoyl-PABA was used as substrate, the assay mixture contained 0.1 ml of enzyme solution, 1.0 ml of 0.1 M sodium phosphate buffer, pH 6.0, 0.1 ml of 80 mM cysteamine hydrochloride, 0.1 ml of 20 mM Na₂EDTA and 0.65 ml of water. After preincubation for 10 min, the enzyme reaction was started by addition of 50 μ l of 30 mM-lipoyl-PABA dissolved in 0.1 M NH₃, which increases the pH of the assay mixture to 6.2. After 30 min, the reaction was stopped by addition of 0.2 ml of 2 M trichloroacetic acid. Immediately after addition of acid, 50 μ l of 10% HSA was added and the protein removed by centrifugation (2500 g, 10 min). The PABA formed was determined in a 1.5 ml portion of the supernatant as described by Knappe et al. [18].

Biotinidase assay

Biotinidase was analysed by spectrofluorimetric determination of lysine released from biocytin, as described by Ebrahim and Dakshinamurti [19]. When this substrate was used, enzyme solution (1.0–2.0 ml) was first dialysed overnight against 1 litre of 50 mM sodium phosphate buffer, pH 6.8, containing 1 mM 2-mercaptoethanol, with one exchange, to remove endogenous primary amines. Similarly, dialysis of samples (1–2 ml) from pooled fractions containing (NH₄)₂SO₄ was necessary because

high NH₄⁺ concentration interfered in the fluorimetric detection of lysine liberated in the enzyme assay. HSA was added after acid precipitation as described for the lipoamidase assay. Otherwise the assay was performed as described by Ebrahim and Dakshinamurti [19].

The enzyme was also assayed by using the artificial substrate biotinyl-PABA. This assay was performed as described for lipoamidase (lipoyl-PABA), except that the substrate was changed to 50 μ l of 30 mM biotinyl-PABA. This substrate concentration is commonly used to measure biotinidase activity [17,18].

Protein determination

Protein was determined as described by Bradford [20], with BSA as calibration standard. Protein determination could not be performed in the different fractions from preparative IEF, since Ampholines interfere with the analysis.

SDS/PAGE

Gel electrophoresis was performed with SDS/12.0%-polyacrylamide gels by the procedure of Laemmli [21], at a constant voltage of approx. 150 V. Gels were stained either with Coomassie Blue R-250 in 50% methanol/7% acetic acid in water or in accordance with the Bio-Rad (München, Germany) silver-staining protocol derived from the method of Merrill et al. [22]. Phosphorylase b, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme (from Bio-Rad) were used as the standard molecular-mass marker proteins.

Starting material

After ordinary analysis, sera sent to our laboratory were pooled to give 1000 ml of starting material. To stabilize the enzyme, cysteine hydrochloride and Na₂EDTA were added to final concentrations of 5 and 1 mM respectively and pH was adjusted to 7.8 with NH₃ immediately before (NH₄)₂SO₄ precipitation. All the following steps were carried out at +4 °C, except that (NH₄)₂SO₄ precipitation was performed in an ice-bath.

Purification procedure

(NH₄)₂SO₄

A solution of 310 ml of 3.8 M (NH₄)₂SO₄, pH 7.8, containing 5 mM cysteine and 1 mM Na₂EDTA, was added to the starting material under gentle stirring to give a final (NH₄)₂SO₄ concentration of 1.6 M. After addition of (NH₄)₂SO₄ the mixture was stirred continuously for another 1 h and then centrifuged at 20000 g for 30 min. The pellet was discarded, and 199 g of solid (NH₄)₂SO₄ was added in portions (final concn. 2.4 M) to the remaining supernatant (1570 ml) under continuous stirring. After centrifugation as above, the pellet was dissolved in 45 ml of 20 mM sodium phosphate buffer, pH 6.8, containing 5 mM cysteine hydrochloride and 1 mM Na₂EDTA (buffer A) to a final volume of 170 ml and dialysed overnight (2 × 5 litres) against the same buffer. After dialysis, the enzyme solution was pooled (280 ml) and divided into two equal portions.

Ion-exchange chromatography

The dialysed solution from the previous step (2 × 140 ml) was loaded on to two DEAE-Sephacel columns (1.6 cm × 35 cm) connected in parallel and equilibrated with buffer A. The columns were washed with 1200 ml of buffer A and the enzyme was then

Table 1 Purification of lipoamidase and biotinidase from human serum

Enzyme activities were measured by both natural and artificial substrates.

Procedure	Volume (ml)	Protein (mg)	Lipoamidase				Biotinidase				
			Substrate ...	Lipoyl-lysine		Lipoyl-PABA		Biotinyl-lysine		Biotinyl-PABA	
				Activity (units)	Specific activity (units/mg or protein)	Activity (units)	Specific activity (units/mg of protein)	Activity (units)	Specific activity (units/mg of protein)	Activity (units)	Specific activity (units/mg of protein)
Human serum	1000	65 000	24 500	0.38	14 480	0.22	10 300	0.16	10 757	0.16	
(NH ₄) ₂ SO ₄ precipitation	280	11 460	16 345	1.4	11 820	0.8	5562	0.5	8565	0.7	
DEAE-Sephacel	416	478	14 327	30	8618	18	5433	11	6683	14	
Blue Sepharose CL-6B	392	284	13 355	47	7699	27	3218	11	6214	22	
Phenyl-Sepharose CL-4B	81	29.4	8590	294	4482	154	2418	83	3967	136	
Isoelectric focusing											
Peak 1	70	0.60	930	1550	435	725	238	397	596	933	
Peak 2	60	6.24	943	151	557	89	362	58	655	105	
Sephacryl S-300 HR											
Peak 1	8	0.04	371	9279	195	4875	128	3200	184	4600	
Peak 2	8	0.14	353	2521	215	1536	173	1236	190	1557	
Total	16	0.18	724	4022	410	2278	301	1672	374	2078	
(peak 1 + peak 2)*											

* Indicates the mean value of the overall purification of peak 1 and peak 2 together.

eluted with a linear NaCl gradient (0–0.2 M) in buffer A at a flow rate of 30 ml/h.

Blue Sepharose CL-6B chromatography

Fractions from ion-exchange chromatography containing specific activity greater than 5 units/mg of protein were pooled (416 ml) and applied to a Blue Sepharose CL-6B column (1.6 cm × 15 cm) previously equilibrated with buffer A. The enzyme was not adsorbed on the column, Flow rate was 30 ml/h, and fractions containing more than 5 units/mg of protein were collected and pooled (392 ml).

Hydrophobic chromatography

Two Phenyl-Sepharose CL-4B columns (1.6 cm × 15 cm) were connected in parallel and equilibrated with buffer B, containing 1 M (NH₄)₂SO₄ dissolved in buffer A. Solid (NH₄)₂SO₄ was added to the pool from Blue Sepharose CL-6B chromatography to give a final concentration of 1 M, and the pool was applied at a flow rate of 30 ml/h on to the phenyl-Sepharose CL-4B columns. The columns were washed with 400 ml of buffer B, followed by elution with a linear gradient of 1 M–0 (NH₄)₂SO₄ (buffer B to buffer A) at a total flow rate of 30 ml/h. Fractions containing specific activity greater than 80 units/mg of protein were pooled and dialysed overnight against buffer A, to obtain 81 ml of enzyme solution.

Preparative isoelectric focusing

For determination of the pI of the enzyme protein, equipment for preparative IEF (LKB 8100 Ampholine column, 110 or 440 ml) was employed in accordance with the methods of Vesterberg and Svensson [23]. Carrier ampholyte in the pH range of 2.5–5.0 and enzyme solution from the previous step were mixed with sucrose to form a density gradient. The column was then subjected to preparative IEF at constant power (10 W) for

72 h at +4 °C in a large column (440 ml); 4.4 ml fractions were collected. When the small column (110 ml) was used, the run was performed at 5 W for 72 h. Since two active fractions, one with pI 4.0 and a second with pI 4.4, were obtained (see the Results section), all four enzyme substrates were used to determine both lipoamidase and biotinidase activities in the fractions. The active fractions from the two peaks which contained lipoamidase activity greater than 10 units/ml were pooled separately, the enzyme activities were measured, and after separate dialysis of the pools against buffer A overnight the enzyme activities were measured again. The enzyme(s) from the two peaks were then concentrated to final separate volumes of 5 ml on Centriflo membrane cones (type CF 50 A) obtained from Amicon Division, W. R. Grace and Co. (Danvers, MA, U.S.A.).

Gel-permeation chromatography

Gel-permeation chromatography was performed on Sephacryl S-300 HR. The concentrated pools from the IEF step were loaded on a Sephacryl S-300 HR column (1.6 cm × 93 cm) previously equilibrated with buffer A. The enzyme was eluted with buffer A, and 2 ml fractions were collected at a flow rate of 30 ml/h. Fractions containing enzyme activity greater than 18 units/ml were pooled.

RESULTS

Purification of lipoamidase and biotinidase in the presence of cysteine as a stabilizer

The results from a representative purification experiment are summarized in Tables 1 and 2. As shown, roughly the same yields and purifications were obtained for lipoamidase and biotinidase, irrespective of which substrate was used for the enzyme determinations. The first four steps all gave rather high yields (54–98 %), and among them ion-exchange chromatography with DEAE-Sephacel gave the highest purification (approx. 20-fold).

Table 2 Summary data for purification of lipamidase and biotinidase by using different procedures and substrates

Procedure	Single step yield (%)				Purification (fold)			
	Lipoamidase		Biotinidase		Lipoamidase		Biotinidase	
	Lipoyl-lysine	Lipoyl-PABA	Biotinyl-lysine	Biotinyl-PABA	Lipoyl-lysine	Lipoyl-PABA	Lipoyl-lysine	Lipoyl-PABA
Human serum	100	100	100	100	1.0	1.0	1.0	1.0
(NH ₄) ₂ SO ₄ precipitation	67	82	54	80	3.6	3.7	3.1	4.4
DEAE-Sephacel	88	73	98	78	21.4	22.5	22.0	20.0
Blue Sepharose CL-6B	93	89	59	93	1.6	1.5	1.0	1.6
Phenyl-Sepharose CL-4B	63	58	75	63	6.2	5.7	7.5	6.2
Isoelectric focusing								
Peak 1	10.8	9.7	9.8	15	5.2	4.7	4.8	6.9
Peak 2	11.2	12.4	15	16.5	0.51	0.58	0.15	0.77
Sephacryl S-300 HR								
Peak 1	40	45	54	31	6.0	6.7	8.1	4.9
Peak 2	37	39	48	29	16.7	17.2	21.3	14.8
Whole procedure								
Peak 1	1.5	1.4	1.2	1.7	24 408	22 159	20 000	28 750
Peak 2	1.4	1.5	1.7	1.8	6634	6982	7723	8482
Total	2.9	2.9	2.9	3.5	10 584	10 355	10 450	12 988
(peak 1 + peak 2)

Figure 1 Preparative IEF on LKB 8100 Ampholine (column 440 ml) in the presence of 5 mM cysteine as stabilizer

After dialysis, the protein obtained from phenyl-Sepharose CL-4B chromatography was loaded on to an apparatus for preparative IEF and run for 72 h with constant power (10 W). The pH gradient is shown as dots. (a) Lipoamidase activity measured with (▲) lipoyl-lysine or (△) lipoyl-PABA as substrate. (b) Biotinidase activity measured with (■) biocytin or (□) biotinyl-PABA as substrate.

Figure 2 Chromatography of lipoamidase on Sephacryl S-300 HR

(a) The protein obtained from peak 1 after preparative IEF (Figure 1) was dialysed and concentrated to 5 ml and applied to a Sephacryl S-300 HR column (1.6 cm × 93 cm) which was equilibrated with buffer A. The protein was eluted with the same buffer. Symbols as in Figure 1. (b) Gel permeation of the protein obtained from peak 2 after preparative IEF (Figure 1) under the same conditions as shown in Figure 2(a). Symbols as in Figure 1.

Figure 3 SDS/PAGE of the purified lipamidase obtained from the two final purification steps

Lane 1, Sephacryl S-300 HR fraction of peak 1 (Figure 2a) (0.31 μ g of protein) from preparative IEF (Figure 1); lane 2, Sephacryl S-300 HR of peak 2 (Figure 2b) (0.81 μ g of protein) from preparative IEF (Figure 1); lane 3, molecular-mass markers. The proteins were developed with Coomassie Blue R-250 stain.

This step was the only one which could separate ceruloplasmin from lipamidase and biotinidase activities.

The material thus obtained was then subjected to preparative IEF. Two peaks were obtained at pI 4.0 and pI 4.4, as shown in Figure 1. We therefore investigated the enzyme activities of all the fractions obtained from preparative IEF and found that the enzyme of pI 4.0 could use both natural and artificial substrates for lipamidase (Figure 1a) and biotinidase (Figure 1b), whereas the enzyme with pI 4.4 could only use the artificial substrates. When the two peaks were separately pooled and dialysed against buffer containing cysteine, the substrate specificity of peak 2 quite unexpectedly changed to include activity also for lipoyllysine and biocytin.

In Table 2 the purification and yield obtained after preparative IEF are reported separately for the two pools after cysteine (re)activation. The total yield of this step was approx. 25%, and the amount of enzyme in the two pools were roughly the same (Table 2), but the specific activity was higher in the pool from peak I (Table 1).

Gel-permeation chromatography of the separate enzyme pools was performed on Sephacryl S 300 HR. As shown in Figure 2, the enzyme activities with all the four substrates remained after gel-permeation chromatography. During this step the enzyme from peak 1 was purified 5–8-fold and peak 2 was purified 15–21-fold (Table 2). With this final step the overall purification was 10000-fold, with a total yield of about 3%.

Figure 4 Preparative IEF (a) and rechromatography by preparative IEF (b) of lipamidase–biotinidase using 2-mercaptoethanol in the first step and cysteine in the second step as stabilizers

(a) A partly purified preparation was obtained as described in the Materials and methods section, except that 2-mercaptoethanol was used as stabilizer in the different steps. After the hydrophobic-chromatography step, the enzyme protein was applied and run on an LKB Ampholine column (440 ml) in the presence of 5 mM 2-mercaptoethanol. Other conditions were as in the experiment shown in Figure 1. Symbols used are as in Figure 1. (b) Cysteine was added to a 25 ml portion of the enzyme pool obtained from purification of enzyme with 2-mercaptoethanol as stabilizer (a) to give a final cysteine concentration of 5 mM, and the enzyme was dialysed overnight against buffer A containing 5 mM cysteine. The enzyme was then applied to the column (110 ml LKB 8100 Ampholine). Symbols are as in Figure 1. (c) Cysteine was added to a 25 ml portion of the enzyme pool as described in (b), except that the final cysteine concentration was 25 mM, and the enzyme was dialysed overnight against buffer A containing 25 mM cysteine. Other conditions were as in the experiment shown in (b). Symbols are as in Figure 1.

SDS/PAGE

To examine the homogeneity and to estimate the molecular mass, the pooled enzyme(s) obtained after gel-permeation chromatography were subjected to SDS/PAGE. As shown in

Figure 3, the protein from the pooled fraction of peak 1 gave a single band with an approximate molecular mass of 76 kDa. The protein from the pooled fraction of peak 2 gave at least two bands on SDS/PAGE, and one of the bands had the same mobility as the protein band from peak 1.

Purification of lipoamidase and biotinidase in the presence of 2-mercaptoethanol as stabilizer

To verify if the enzyme in peak 2 obtained after preparative IEF was due to an artifactually produced 'cysteine adduct', we purified the enzyme in the presence of 2-mercaptoethanol as a stabilizer instead of cysteine. Otherwise the purification was performed as described above, but lipoyl-PABA was used as the only substrate for enzyme determination until preparative IEF. Similar purification was obtained (results not shown) as in the purification reported above, but, as shown in Figure 4(a), only one major peak, with pI 4.0, was obtained after preparative IEF. After dialysis against 5 mM cysteine a portion of the pooled fraction obtained from this peak was re-chromatographed by preparative IEF. Two peaks were obtained (Figure 4b), one peak with pI 4.0 and another at pI 4.4. When a portion of the dialysed enzyme containing 5 mM cysteine was supplemented with cysteine to a final concentration of 25 mM and re-chromatographed on preparative IEF, only one peak, at pI 4.4, was obtained (Figure 4c).

DISCUSSION

Recently we described low lipoamidase activity in serum from a patient with biotinidase deficiency [24]. This, together with findings by other authors, indicated that serum biotinidase and lipoamidase might be one of the same enzyme. In the present study we have purified lipoamidase from human serum, and, as shown in Table 1, purification and yield of the two enzyme activities were very similar in all purification steps. There is only one earlier report on lipoamidase purification from human serum, which gave a 983-fold purification with lipoyl-PABA as substrate [15]. Human serum biotinidase has been purified to homogeneity [16,25,26], with a reported molecular mass of 68 [25,27], 67 [26] and 76 kDa [16]. The finding of similar molecular mass of our lipoamidase/biotinidase is in line with the view that the two enzymes in human serum are confined to the same protein.

Garganta and Wolf [17] reported that lipoamidase activity was lost (> 95%) without affecting the biotinidase activity during gel chromatography, but the lipoamidase activity could be re-activated by dithiothreitol. This indicated that the enzyme is sensitive to oxidation of some cysteine residue in or near the active site in a more complex structure, because the enzyme activity was not inactivated when biotinyl-PABA was used as the substrate.

Lipoamidase from human serum is stimulated by thiol compounds and inhibited by thiol reagents [14]. Therefore it should be stabilized by a thiol compound and EDTA. We used cysteine because it is an endogenous thiol compound in human blood and not so expensive as, e.g., glutathione. 2-Mercaptoethanol is frequently used as a thiol stabilizer, but unfortunately it could not be used in our purification, and neither could dithiothreitol or other uncharged thiols, because they interfered with the Ellman reaction used in the lipoate analysis. Only aminothiols compounds (such as cysteine) can easily be used in the enzyme assay [14], because they are completely separated from lipoic acid during the extraction before the Ellman reaction. Biotinidase

from serum is also stimulated by thiol compounds [27,28], but, in contrast with lipoamidase, it was not necessary to stabilize the enzyme during the purification procedure [16,25,26].

After preparative IEF the enzyme was separated in two fractions with different pI values, as shown in Figure 1. Further, these fractions also differ in substrate specificity. The enzyme obtained from peak 1 (pI 4.0) can split all substrates tested, in contrast with the enzyme obtained from peak 2 (pI 4.4), which could hydrolyse only the synthetic substrates for both lipoamidase and biotinidase. These results indicated that the enzyme in peak 1 obtained after preparative IEF is similar to biotinidase.

The pI of human serum lipoamidase has not been described previously, and for human serum biotinidase it varies from 4.6 [16], 4.3, range 4.2–4.4 [29], to 4.15–4.35 [30], which should be compared with our results of 4.0. The presence of cysteine during the preparative IEF resulted in two separate peaks with different pI values. Thus the results with various concentrations of cysteine added to enzyme pools obtained by purification with 2-mercaptoethanol as stabilizer indicate that cysteine at high concentration changes the enzyme protein to give a higher pI. If cysteine binds to a free thiol group or splits an intramolecular S-S bridge, the results would give a different folding of the protein and accordingly a different pI of the enzyme protein.

We concluded that lipoamidase and biotinidase have been co-purified more than 20000-fold from human serum. The protein preparation obtained was homogeneous as found by SDS/PAGE, and had an approximate molecular mass of 76 kDa. With high concentrations of cysteine in the purification procedures conformational changes probably occur, as found by changes in pI and in substrate specificity of both lipoamidase and biotinidase. This further strengthens the argument that lipoamidase and biotinidase activities are confined to the same enzyme protein in human serum.

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