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SEPARATION AND CHARACTERIZATION OF TWO ISOLATED LIPASES FROM *STAPHYLOCOCCUS AUREUS* (TEN5)

JOCHEM KÖTTING*, DAGMAR JÜRGENS and HANS HUSER
Robert Koch-Institut des Bundesgesundheitsamtes, Nordufer 20, 1000 Berlin 65 (F.R.G.)
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SUMMARY

The purified lipases from *Staphylococcus aureus* (TEN5) showing two enzymatically active protein bands on SDS–polyacrylamide gel electrophoresis have been separated by ion-exchange chromatography. The separated proteins show some properties which are different (*e.g.*, apparent molecular weight, charge, binding of detergent, enzymatic activity towards triolein) and some which are almost identical (spur in immunodiffusion).

INTRODUCTION

Microbial lipases are of considerable importance in industrial processes and in the diagnosis of various infectious diseases. Many purification procedures and characterizations have been performed[1]. This is especially true with respect to staphylococcal lipases[2–10]. As these partially purified enzymes were not rigorously tested for their molecular homogeneity, little can be said about their properties[2–9]. The characteristics of lipases from bacteria differ even when these enzymes are obtained from bacteria of the same species[10].

Jürgens *et al.*[11] purified lipases from *Staphylococcus aureus* (TEN5)[12] to constant specific activity (SA). It was shown earlier that this lipase preparation consisted of two lipolytic polypeptides with apparent molecular weights (M_r) of 43 and 44 kD (kilodaltons), respectively.

In order to look at the differences in these proteins, they were separated by ion-exchange chromatography. Their physico-chemical, immunological and enzymatic properties were studied.

EXPERIMENTAL

Bacterial strain

Staphylococcus aureus (TEN5) was isolated and recognized as an effective lipase producer by Arbuthnott *et al.*[12]. The strain was a kind gift from Dr. C. G. Gemmell, Bacteriology Department, University of Glasgow, Royal Infirmary, Glasgow, U.K.

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Production and purification of lipases

These were performed by use of hydrophobic interaction chromatography (HIC) in the last step as described by Jürgens and Huser[13].

Protein determination and lipase activity measurement

Protein concentration was determined as described by Petersen[14] with bovine serum albumin as a standard. Lipase was measured with a pH-stat technique[15]. The substrate dispersion was prepared in distilled water and contained triolein (Serva, Heidelberg, F.R.G.) (5 m*M*), gum arabic (Merck, Darmstadt, F.R.G.) (5%, w/v), DOC (sodium desoxycholate) (Merck) (4 m*M*) and sodium chloride (0.1 *M*). Released oleic acid was titrated continuously with 0.02 *M* sodium hydroxide concentration at 30°C. One unit of lipase (1 U) is defined as that amount of lipase liberating 1 μM/min of oleic acid.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

This was performed according to Laemmli[16]. A 10–40-μg amount of protein per trough was loaded. Phosphorylase (M_r 95 kD; E.C. 2.4.1.1), bovine serum albumin (M_r 67 kD), fumarase (M_r 49 kD; E.C. 4.2.1.2), carboanhydrase (M_r 30 kD, E.C. 4.2.1.1) and lysozyme (M_r 14 kD; E.C. 3.2.1.17) were used as reference proteins and purchased from Boehringer (Mannheim, F.R.G.).

Molecular weight determination by gel filtration on Sepharose CL-4B

The gel filtration was performed on Sepharose CL-4B (Pharmacia, Uppsala, Sweden) equilibrated with a 0.1 *M* ammonium hydrogen carbonate (pH 8.0) solution containing 0.2 *M* sodium chloride. The column (130 × 1.2 cm I.D.) was calibrated with the following proteins: aldolase (E.C. 4.1.2.13; M_r 158 kD), catalase (E.C. 1.11.6; M_r 232 kD), ferritin (M_r 440 kD) and thyroglobulin (M_r 669). The void volume was determined with Blue Dextran 2000. The flow-rate was 5 ml/h and the fraction volume was 1.2 ml. Protein was detected at 280 nm.

A Triton-free lipase preparation (containing both lipases, 0.5 mg; SA 1000 U/mg) was applied in 0.5 ml of ammonium hydrogen carbonate solution to the column and eluted as described above. Lipase in the eluate was detected by lipolytic activity measurements. Triton was removed according to Holloway[17]. It was quantitated as described by Garewal[18].

Separation of lipases by cation-exchange chromatography

The HIC purified lipase preparation (5 mg; SA 1000 U/mg) was applied to a column (20 × 1 cm I.D.) of SP-Sephadex C-50 equilibrated with 0.067 *M* phosphate buffer (pH 7.0). The column was eluted with 50 ml of phosphate buffer (0.067 *M*) (pH 7.0) and 50 ml phosphate buffer (0.067 *M*) (pH 8.2). The last elution volume consisted of 50 ml 0.2 *M* sodium carbonate–sodium hydrogen carbonate buffer (pH 10.0). The flow-rate was 6 ml/h. Fractions of 1 ml were collected.

Protein titration curve

The titration curves were measured in a slab gel containing 5% polyacrylamide as described by Righetti *et al.*[19].

Preparation of lipase anti-sera

To produce antibodies, 0.5 mg of each of the two separated lipases were mixed thoroughly with 1 ml of Freund's complete adjuvant (Difco Labs., Detroit, MI, U.S.A.) and injected into rabbits subcutaneously. Similar injections were given in the fifth and sixth weeks. One week after the last injection blood was drawn from the ear of each rabbit. The sera obtained were stored at −20°C and labelled either anti-43 kD serum or anti-44 kD serum.

Immunodiffusion

Immunodiffusion was performed as described by Ouchterlony and Nilsson[20].

Charge-shift immunoelectrophoresis

Charge-shift immunoelectrophoresis was performed as described by Helenius and Simons[21].

RESULTS

Molecular weight determination by gel chromatography

Earlier M_r determinations under detergent-free conditions in Sephadex G-200 gave results for the impure *S. aureus* lipase ranging from 200 to 1000 kD[6,22]. Gel filtration of a pure detergent-free lipase preparation (still containing two lipolytically active polypeptides) on Sepharose CL-4B revealed one defined lipase aggregate with an M_r of only 780 kD ± 5% (Fig. 1).

Separation of lipases

Batch experiments showed that the two lipases bound to weak and strong cation exchangers. However, both polypeptides were eluted simultaneously from the weak cation exchanger. With the strong cation exchanger SP-Sephadex C-50, stepwise elution of the two lipases could be used, as follows. A 5-mg amount of an HIC

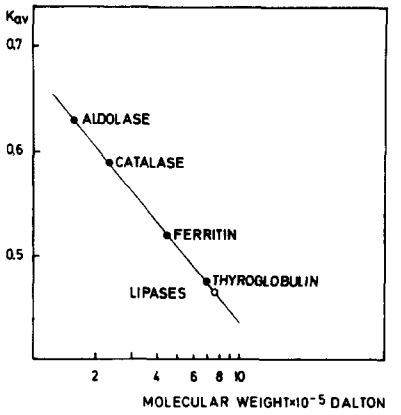

Fig. 1. Determination of the apparent molecular weight of lipases on Sepharose CL-4B. Semilogarithmic plot of the partition coefficient (K_{av}) *versus* apparent molecular weight (M_r) of calibration proteins. Lipases (M_r 780 kD); calibration proteins, aldolase (M_r 158 kD), catalase (M_r 232 kD), ferritin (M_r 440 kD), thyroglobulin (M_r 669 kD).

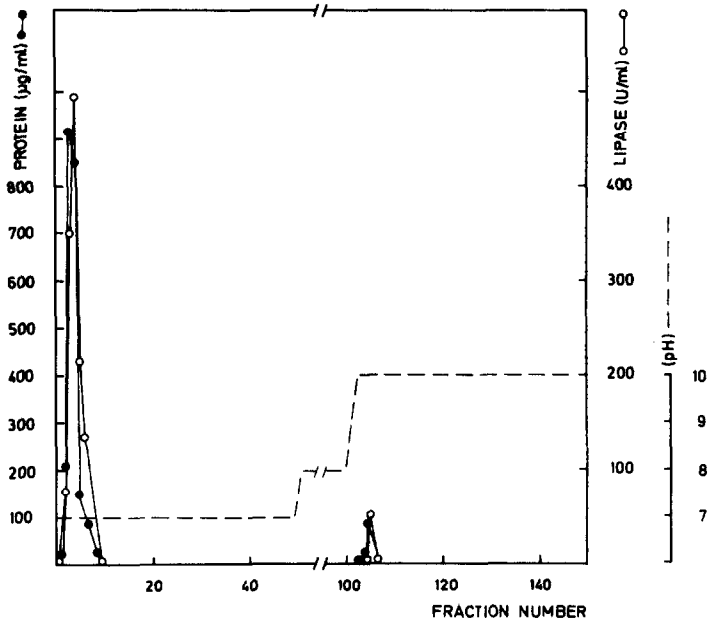

Fig. 2. Ion-exchange chromatography of lipase preparation on SP-Sephadex C-50 cation exchanger: 5 mg of lipase preparation (SA 780 U/mg) were applied on a column (20 × 1 cm) equilibrated with 0.067 *M* phosphate buffer (pH 7.0). Stepwise elution: (1) with a five-fold column volume of 0.067 *M* phosphate buffer (pH 7.0); (2) with a five-fold column volume of 0.067 *M* phosphate buffer (pH 8.2); (3) with 0.2 *M* sodium carbonate–sodium hydrogen carbonate buffer (pH 10.0). 150 fractions to 1 ml/fraction were collected at a flow-rate of 15 ml/h. -○-○-, Lipase activity: -●-●-, protein concentration; ---------, pH.

purified lipase preparation (SA 1000 U/mg) (Fig. 3, position 3) in 1 ml of phosphate buffer (0.067 *M*) (pH 7.0) was applied to an SP-Sephadex C-50 column. The column was washed with the same buffer which eluted a lipase in fractions 3–10 (Fig. 2). SDS–PAGE showed it to be the 43 kD lipase (Fig. 3, positions 4 and 6). The column was then rinsed with 50 ml of phosphate buffer (0.067 *M*) (pH 8.2). This rinse increased the purity of a second lipase polypeptide which was eluted with 50 ml of 0.2 *M* sodium carbonate–sodium hydrogen carbonate buffer (pH 10.0). Fractions 104–106 (Fig. 2) contained, as shown by SDS–PAGE, the pure 44 kD polypeptide (Fig. 3, positions 5 and 7). The yield of the purification is shown in Table I.

Titration curves

To investigate the behaviour of the lipase over a broad pH range, a combination of isoelectric focusing in the first dimension and electrophoresis in the second dimension was employed[19]. Fig. 4 shows that both lipases have an identical isoelectric point of pH 9.9 ± 0.2. In titrating the isolated 43 kD polypeptide it could be shown that it corresponded to the slower migrating protein band. There are migration differences between the two lipases from pH 7 to 4, whereas the two curves overlap from pH 7.5 to 10.

Immunological characterization

The antibodies raised in rabbits were tested in double diffusion according to

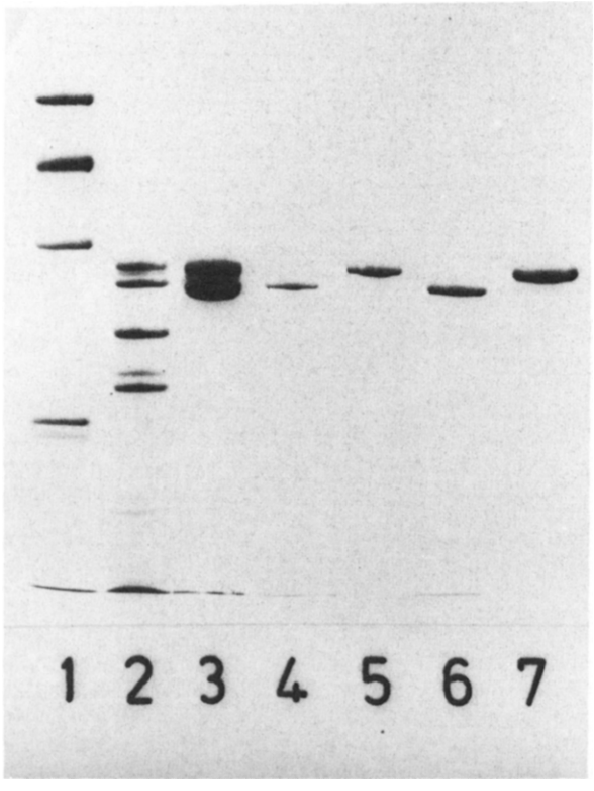

Fig. 3. SDS–PAGE of the lipase fractions from cation-exchange chromatography (*cf.*, Fig. 2): (1) 3–5 μg of each of the marker proteins; (2) about 100 μg of the ammonium sulphate-precipitated material; (3) HIC-purified lipase, 20 μg of protein, starting material; (4) 5 μg of protein of fraction 3 (Fig. 2) corresponding to the 43 kD lipase; (5) 5 μg of protein of fraction 105 (Fig. 2) corresponding to the 44 kD lipase; (6) and (7) as positions 4 and 5, respectively, but twice as much protein was applied. Proteins were stained with Coomassie Brilliant Blue.

TABLE I

YIELD OF LIPASES BY CATION-EXCHANGE CHROMATOGRAPHY ON SP-SEPHADEX C-50

	Protein (mg)	*Yield (%)*	*Activity (U)*	*Yield (%)*	*SA (U/mg)*
Applied	5	100	3900	100	780
Elution step:					
pH 7.0	2.5	50	1280	33	560
pH 8.2	Traces	–	–	–	–
pH 10.0	0.2	4	102	3	530
Total	2.7	54	1382	36	

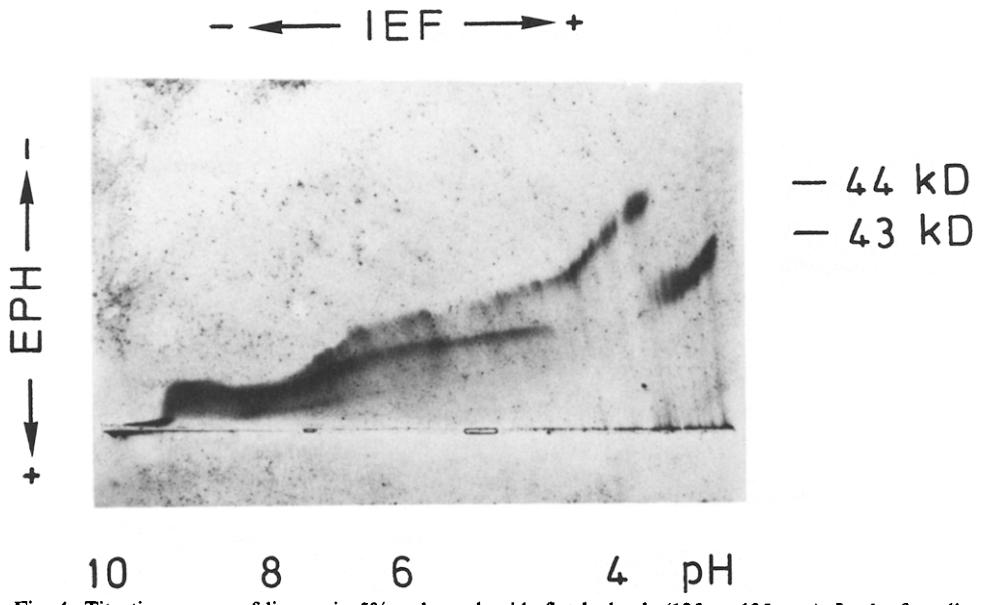

Fig. 4. Titration curves of lipases in 5% polyacrylamide flat bed gels (125 × 125 mm). In the first dimension isoelectric focusing (IEF) in the pH range 11–2 (1 h, 10 W) was performed. For the second dimension electrophoresis (EPH): 100 µl of a lipase preparation (SA 2000 U/ml; protein concentration 1 mg/ml; 10 min, 800 V) were added in the middle trough. Proteins were stained with Coomassie Brilliant Blue.

Ouchterlony and Nilsson[20]. Fig. 5 reveals partial immunological identity but also differences (spur).

The catalytic activity of the lipases was completely inhibited by incubating them with the corresponding antisera (12 h, 37°C). The anti-44 kD serum was also able to inhibit the 43 kD lipase activity and *vice versa*.

Immunoelectrophoresis at pH 8.7 in the presence of Triton X-100 showed a greater electrophoretic mobility for the isolated 44 kD lipase than for the 43 kD lipase. The 43 kD polypeptide stayed at the origin (Fig. 6a). In Triton-containing

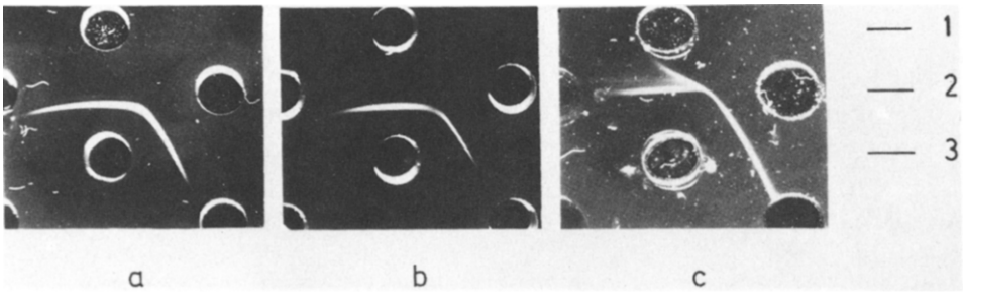

Fig. 5. Immunodiffusion of lipase according to Ouchterlony and Nilsson[20]: well 1, 43 kD, 10 µg of protein were applied; well 2, 44 kD, 10 µg of protein were applied; well 3a, anti-43 kD serum; well 3b, anti-44 kD serum; well 3c, anti-43 kD serum and anti-44 kD serum, 5 µl of each were applied. Agarose 1%; 0.02 *M* phosphate buffer (pH 7.3); 0.9% (w/v) sodium chloride; diffusion for 48 h.

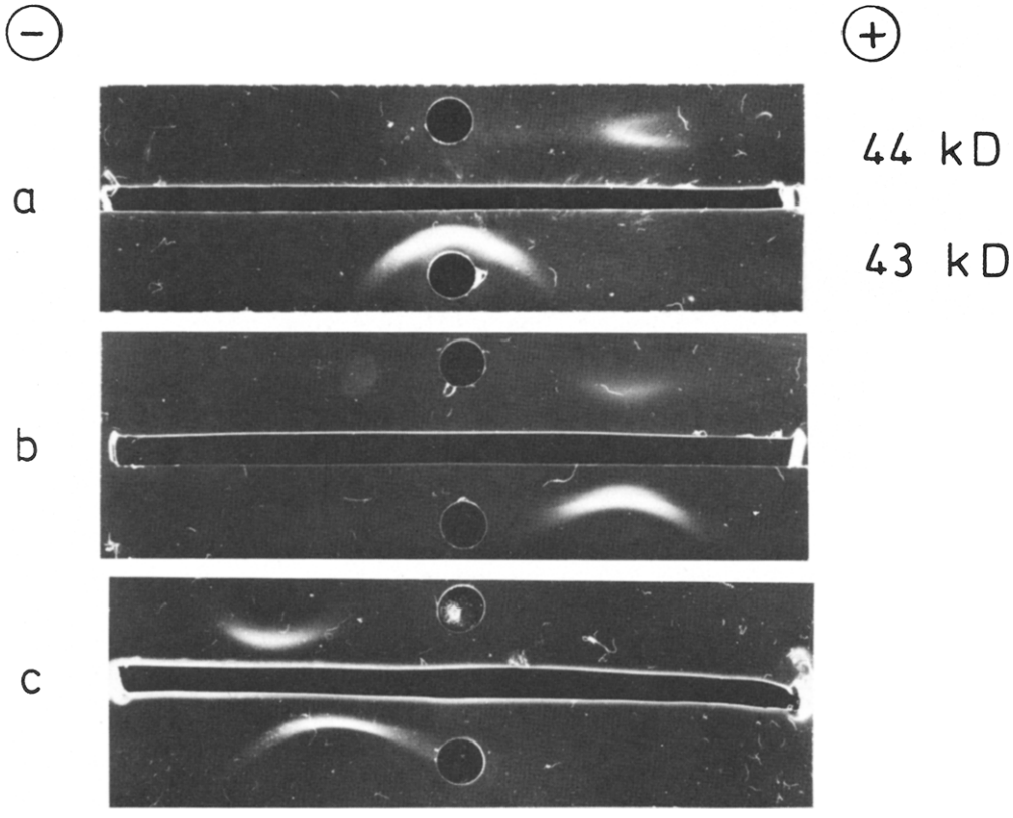

Fig. 6. Characterization of lipases in charge-shift immunoelectrophoresis. Electrophoresis was performed in 1% agarose gels in the presence of 0.1 *M* glycine–0.038 *M* Tris buffer (pH 8.7). Different detergents were added: (a) 0.5% (w/v) Triton X-100 in agarose, no detergent in buffer; (b) 0.5% (w/v) Triton X-100 and 0.2% (w/v) sodium desoxycholate in gel and buffer; (c) 0.5% (w/v) Triton X-100 and 0.0125% (w/v) cetyltrimethylammonium bromide in gel and buffer. In each of the upper holes in a, b and c, 10 μg of the 44 kD lipase were applied. Each of the lower holes was filled with 10 μg of the 43 kD lipase. Electrophoresis was performed for 4 h at 80 V and 10°C. For detection 100 μl of anti-44 kD serum were placed in the central well.

gels distinct differences with respect to the electrophoretic mobility of the isolated lipases are obvious (Fig. 6a).

Charge-shift immunoelectrophoresis

Fig. 6b shows an identical mobility of both lipases in the presence of the anionic detergent DOC. When cetyltrimethylammonium bromide (a cationic detergent) was present, both lipases migrated towards the cathode (Fig. 6c).

The mobilities were slightly different. The lipases were detected with either anti-43 kD serum or anti-44 kD serum. This different migration behaviour in the presence of differently charged detergents is typical of amphiphilic proteins[21].

Hydrolysis of triolein

The K_m values of the separated lipases were determined using triolein as the

TABLE II

MICHAELIS-MENTEN CONSTANTS OF THE *S. AUREUS* LIPASES WITH TRIOLEIN AS SUBSTRATE

Parameter	*43 kD lipase*	*44 kD lipase*
K_m (mM)	1.6 ± 0.2	1.1 ± 0.1
V_{max} (U/mg)	1250 ± 160	100 ± 13

substrate. The reaction velocity was measured in a pH-stat according to Rick[15]. Starting from a 5 mM triolein emulsion in 10% (w/v) gum arabic, various concentrations with respect to triolein (to 0.313 mM) were made by dilution with 0.4 M sodium chloride solution containing 0.66% (w/v) DOC. Evaluation was carried out according to the Lineweaver–Burk method and the values are listed in Table II. Both the K_m and V_{max} values are different for the two isolated lipases.

DISCUSSION

In contrast to earlier findings on impure *S. aureus* lipase, which showed an aggregation weight of 200–1000 kD[6,22]; the material of constant SA (still containing two lipolytic polypeptides of 43 and 44 kD) revealed a distinct molecular weight of 780 kD ± 5%. The broad aggregation weight of the impure lipases could be due to the presence of other lipophilic substances, as suggested earlier[23]. Our findings of a distinct molecular weight of 780 kD of the purified lipases would exclude such impurities and show a well defined aggregated state. An aggregation number of 18 can be calculated if an average molecular weight of 43.5 kD for the two lipolytic polypeptides was assumed.

A successful separation on a cation exchanger is limited in the active range by the precipitation of the lipases below pH 7. Above pH 7 their charge properties are very similar, as can be seen also in the titration curves (Fig. 4). The low yield of the 44 kD polypeptide in Table I in our opinion results from non-specific adsorption to the cation exchanger. In trying to increase the yield of the 44 kD lipase, on SDS–PAGE it was always observed that 43 kD material was present. Therefore, only the purest preparations of the 44 kD lipase were used for further investigations.

The first differences amongst these isolated lipases were found in their apparent molecular weights (Fig. 3, positions 4 and 5) and their different electrophoretic mobilities (Figs. 4 and 6a). The charge-shift immunoelectrophoresis (Fig. 6) characterized both lipases as amphiphilic proteins[21]. Whereas the 43 kD lipase bound anionic and cationic detergents to the same extent, the 44 kD lipase migrated the same distance in the presence and absence of DOC.

In comparison with observations with pancreatic colipase, which does not bind positively charged desoxycholate derivatives[21], it might be that the 44 kD lipase does not bind the anionic DOC under these experimental conditions.

Further differences were observed in the catalytic properties towards triolein. The K_m values are, however, apparent K_ms as the water-insoluble substrate was sonicated with detergent. Enzymatic hydrolysis occurred at the droplets' interface. The Michaelis–Menten kinetics may be applied formally if it is kept in mind that the size

of the droplets influences the K_m values[1].

The differences found in this investigation should be further substantiated with stereospecific substrates, amino acid analysis and peptide maps.

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