CHAPTER 7

Screening of Commercial Lipases for the Degradation of Ochratoxin A

This project was done at the Institut für Technische Biochemie, Universität Stuttgart, Stuttgart, Germany, under supervision of dr. Uwe Bornscheuer, preliminary screenings were done by mr. Erik Henke. The results of **Chapter 7** will be submitted for publication in 2000. The aim of the project was to find a commercial lipase that possesses the ability to degrade OTA and that can possibly be used in the decontamination of OTA contaminated foods.

Part 1: Screening of Commercial Lipases for the Degradation of Ochratoxin A

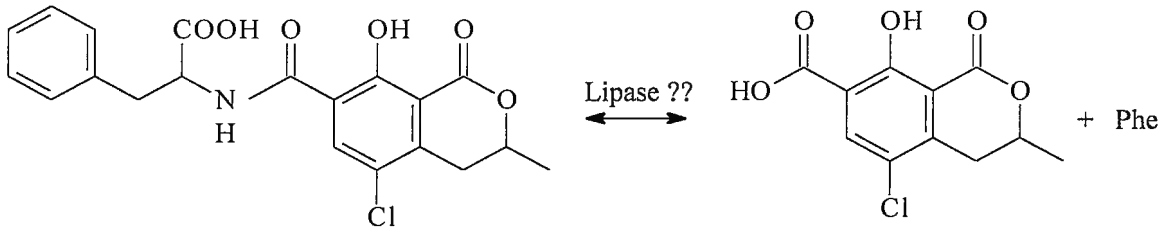
ABSTRACT

Ochratoxin A is a nephrotoxic and carcinogenic mycotoxin. The toxin is a common contaminant of various foods and feeds and pose a serious threat to the health of both humans and animals. A number of commercial lipases were screened for the ability to degrade OTA to non-toxic compounds. The lipase from *Aspergillus niger* from the supplier, Amano proved to substantially hydrolyse OTA to the non-toxic OTα and phenylalanine, as illustrated by HPLC with fluorescence detection.

INTRODUCTION

Ochratoxin A (OTA) is a nephrotoxic, teratogenic, hepatotoxic and carcinogenic mycotoxin that is the cause of diseases in animals including Danish porcine nephropathy and has been implicated in the etiology of diseases in humans (Balkan endemic nephropathy and urinary tract tumours) [Reviews: Marquardt and Frohlich, 1992, Steyn and Stander, 1999]. The toxin is produced by a number of *Penicillium* and *Aspergillus* species and is a common contaminant in foods including coffee, beer, wine, grains and spices (van Egmond and Speijers, 1994). OTA has been identified in the blood of humans in a number of countries after the consumption of contaminated food (Petkova-Becharova *et al.*, 1988; Breitholtz *et al*, 1991; Creppy *et al.*, 1991; Bacha *et al.*, 1993). OTA is a very stabile compound that can only be completely hydrolysed by heating under reflux for 48 hours in 6 M hydrochloric acid (van der Merwe *et al.*, 1965). The mammalian enzyme, carboxypeptidase A has the ability to cleave OTA (Pitout, 1969; van der Westhuizen *et al.*, 2000). The hydrolysis products are phenylalanine and the non-toxic, ochratoxin α (OTα).

Lipases are known to cleave amide bonds (Bornscheuer and Kazlauskas, 1999) and are used to deprotect peptides (Braun *et al.*, 1990) in commercial processes and in biocatalytic resolution of e.g. (±)-trans-cyclohexane-1,2-diamine (Alfonso *et al.*, 1996). In an effort to find viable ways to decontaminate OTA contaminated foods, and to study possible structural/function relationships of ochratoxins and related substances in lipase reactions, 23 commercial lipases were screened for OTA hydrolysis.



Ochratoxin A Ochratoxin α

Figure 1: Structures of OTA and OTα showing the schematic effect of lipase

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Fluka (Buchs, Switzerland) and Sigma (Steinheim, Germany) at the highest purity available. The lipases were supplied by the different companies indicated in **Table 1.** OTA was extracted from Durum wheat inoculated with *Aspergillus ochraceus* (Stander *et al.*, 1999). OTα was produced by hydrolysing OTA under reflux in excess 6 M hydrochloric acid for 60 hours.

HPLC

A Waters 2690 system fitted with a fluorescence detector and a photodiode array detector, termostated column compartment, autosampler and Millenium software was used. The excitation wavelength was set at 250 nm and the emission wavelength at 454 nm. Separation was achieved on a C_{18} reversed phase column (4.6 mm × 150 mm, 5 μm, Discovery, Supelco, Bellefonte, PA, USA) at 30 °C, employing an isocratic mobile phase of methanol/water/acetic acid (60:50:2), and a flow rate of 1 ml/min.

Screening for enzymes that degrade OTA

OTA was dissolved in toluene and stirred with the enzyme (+/- 10 mg, see the enzymes that were screened in **Table 1**) in phosphate buffer (pH 7.5, 50 mM, 1 ml) for 18 hours at 37 °C. The degradation of OTA was monitored with TLC on silica plates (toluene/acetic acid, 4:1, OTA: R_f 0.5; OTα: R_f 0.23). Only the lipase from *Aspergillus niger* showed degradation of OTA to possibly OTα, no degradation was observed in the other enzyme reactions.

Table 1: Enzymes screened for OTA degradation

	Enzyme	**Organism**	**Source**
1	Lipase D (LDW05525)	*Rhizopus delemar*	Amano
2	Lipase R (LRFS 10520)	*Penicillium roquefortii*	Amano
3	Lipase F-AD 15 (LFS 06522)	*Rhizopus javanicus*	Amano
4	Lipase A (LSO6507)	*Aspergillus niger*	Amano
5	SP 525 (PPW 5007)	*Aspergillus oryzae*	Novo Nordisk
6	Lypozyme 1M (LUX 110)	*Rhizomucor miehei*	Novo Nordisc
7	Lipase F (NSO4529)	*Rhizopus javanicus*	Amano
8	Lipase AH (LPAHQ001K)	*Pseudomonas cepacia*	Amano
9	ACS (ACS02525)	(Acylase)	Amano
10	Lipase G (LGDS 01507)	*Penicillium camembertii*	Amano
11	AC 405 (EAC 080120)	(Acid esterase)	Amano
12	Lipase PUUR (0026/MV4059)	*Pseudomonas alkaligenes*	Gist Brocades
13	CAL B (Chirazyme L-2,c.-f.,C3,lyo)	*Candida antarctica B*	Boehringer Mh..
14	PFE	*Pseudomonas fluorescens* (esterase)	
15	Lipase AY (LAYV07530) Amano 30	*Candida rugosa* resp. *C. cylindracea*	Amano
16	Lipase AYS (CRL) (LAYW03506S)	*Candida rugosa*	
17	Lipase pure	*Chromobacterium viscosum*	Asahi
19	*Humicola lanuginosa*	*Humicola lanuginosa*	Biocatalyst
20	LMR 05525		Amano
21	CAL-B SP 525	*Candida antartica B*	Novo
22	CAL-A SP 526	*Candida antartica A*	Novo
23	PCL	*Burkholdia cepacia;* earlier *Pseudomonas cepacia*	Boehringer Mannheim

The determination of the activity of the lipase from A. niger

The lipase from *A.niger* (5 ml, 78.6 μg/ml in 20 mM Tris/HCl, pH7.5) was stirred with OTA (0.49 mg in 1 ml toluene) at 37 °C. The reaction was done in 18-fold (9 experiments stopped at different time intervals in duplicate). The reaction was stopped (in duplicate) after 0, 5, 10,

15, 20, 30, 60, 90, and 120 minutes by adding 1 M HCl (1 ml). The organic compounds were extracted with ethyl acetate (5 ml), the solvent (1 ml) was evaporated under a stream of dry nitrogen, resuspended in methanol (1 ml) and injected as 4 µl aliquots onto the HPLC.

RESULTS AND DISCUSSION

Lipase A from *Aspergillus niger* (Amano) was the only lipase that showed degradation of OTA (See **Figures 2-4**). OTA was substantially hydrolysed to phenylalanine and OTα. The activity of the lipase might be attributed to the fact that some *Aspergillus niger* strains are known to produce OTA (Abarca *et al.*, 1994).

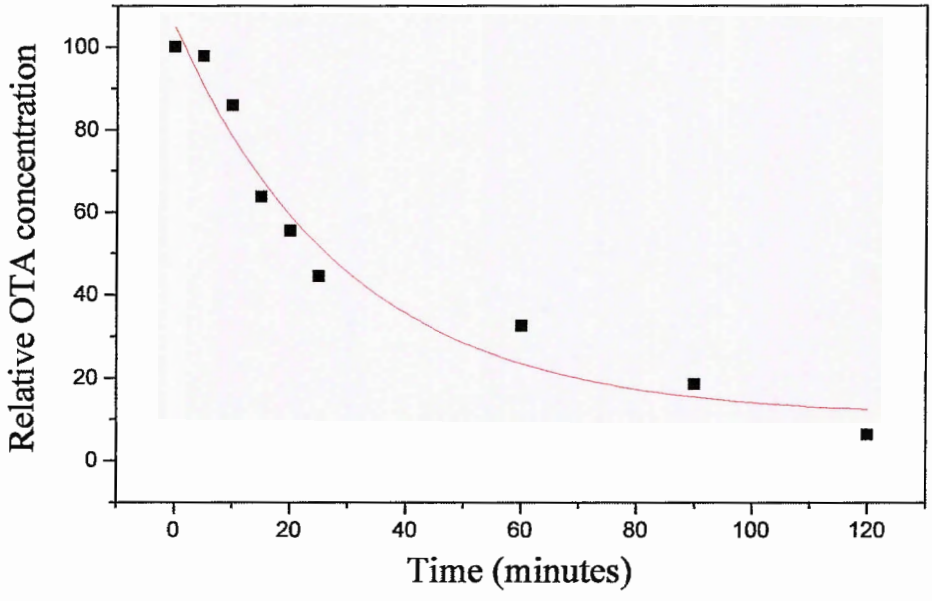


Figure 2: The hydrolysis of OTA by the lipase from *Aspergillus niger*: Relative OTA concentration versus time.

The HPLC analysis confirmed that OTA was degraded to OTα (UV and retention time comparison) by the lipase from *Aspergillus niger*. One unit of lipase activity is defined as the amount of enzyme that liberates 1 µmol fatty acid per minute and was calculated to correspond to 131 mg enzyme that liberates 1 µmol of OTA per minute. The activity was

determined by using an HPLC-calibration curve for OTA (y = 3370x, with a correlation coefficient of 0.9993) and calculating the slope of the linear region (0-35 min) of the OTA concentration versus time curve. The results clearly indicate that the lipase from *Aspergillus niger* can be used to degrade OTA substantially. Further research needs to be done in implementing it in a decontamination process to remove OTA from OTA-contaminated foods and feeds.

ACKNOWLEDGEMENTS

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Supporting information

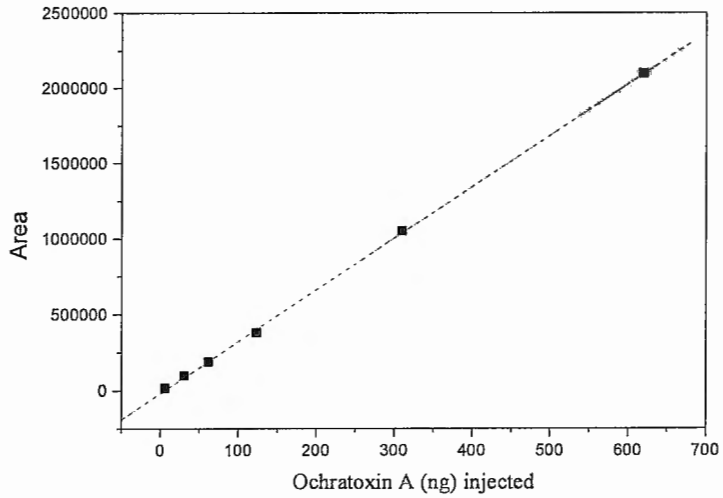


Figure 5: Calibration curve of OTA on HPLC with fluorescence detection (y = 3370x; R^2 = 0.9993).

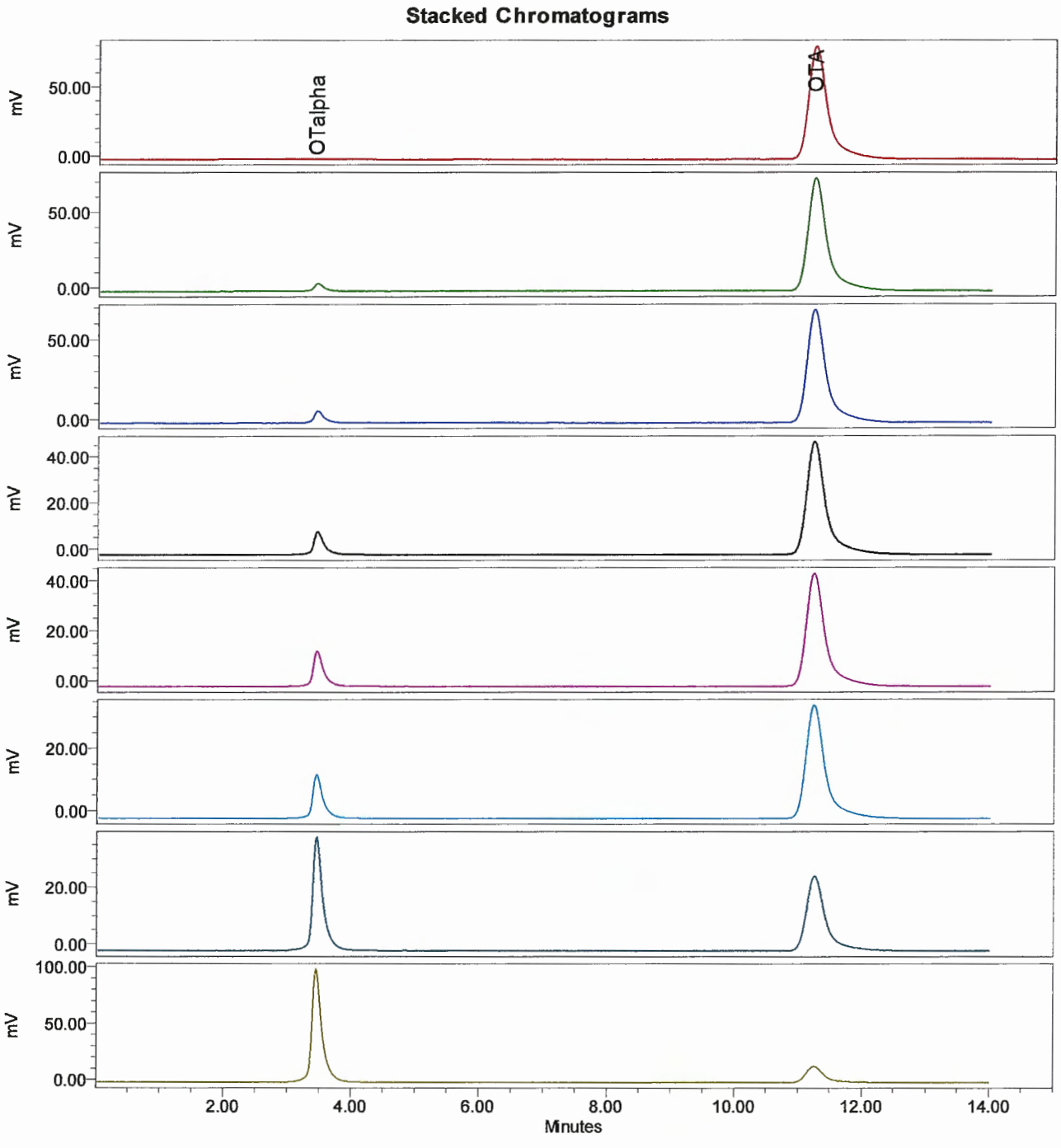


Figure 3: Stacked HPLC-chromatograms of the hydrolysis of OTA by the lipase of Aspergillus niger, showing a decrease in the OTA concentration and increase in the ochratoxin alpha concentration after different reaction time intervals.

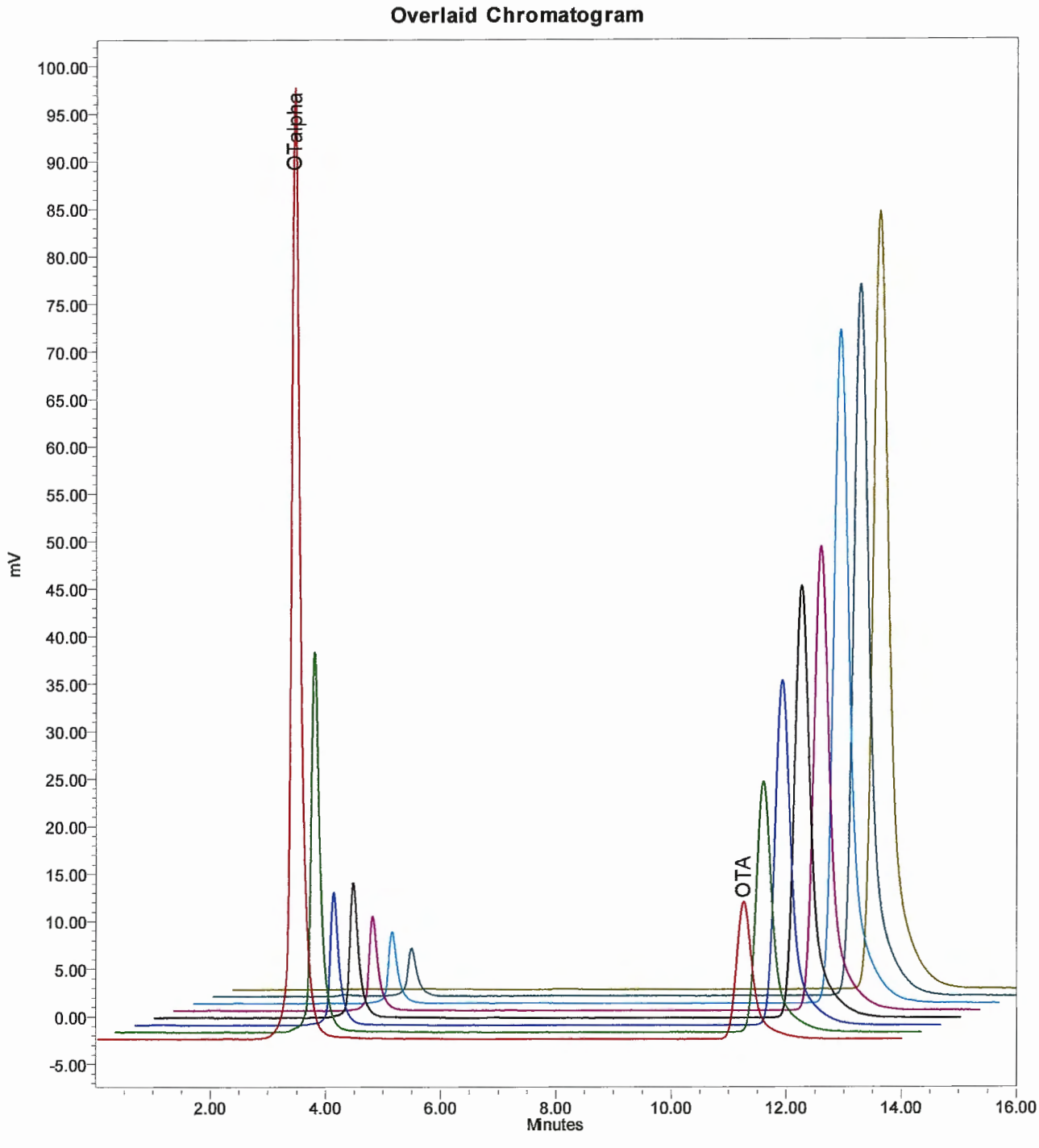


Figure 4: Angled overlay HPLC-chromatograms of the hydrolysis of OTA by the lipase of Aspergillus niger, show ing a decrease in the OTA concentration and increase in the ochratoxin alpha concentration after different reaction time intervals.

Part 2: Efforts to purify the commercial lipase from *Aspergillus niger*

INTRODUCTION

In **Part 1** a number of commercial lipases were screened for OTA degradation. A commercial lipase isolated from *Aspergillus niger* obtained from Amano was found to substantially hydrolyse OTA to the non-toxic OTα and phenylalanine. This lipase was not previously characterised and therefore not much is known about its characteristics. It was decided to purify the hydrolytically active enzyme to determine its activity and its N-terminal sequence.

MATERIALS AND METHODS

SDS electrophoresis

Solutions

Lower Tris: Tris (36.34 g) and SDS (0.8 g) were dissolved in water (150 ml) and the pH adjusted to 8.8 with 1 M hydrochloric acid, whereafter water was added to a total volume of 200 ml.

Upper Tris: Tris (12.11 g) and SDS (0.8 g) were dissolved in water (150 ml) and the pH adjusted to 8.8 with 1 M hydrochloric acid, whereafter water was added to a total volume of 200 ml.

SDS buffer solution for sample preparation: Glycerol (10 ml), 2-mercaptoethanol (5 ml), SDS (3 g), bromo phenol blue solution (2.5 ml, 0.05 % (m/v) in water) and upper Tris (12.5 ml) were made up to a total volume of 50 ml.

Electrode buffer: Tris (3 g, 0.025 M), glycine (14.4 g, 0.192 M) and SDS (1 g, 0.1%) were dissolved in water (1 l) and the pH was adjusted to 8.4.

Sample preparation for SDS electrophoresis

The protein samples (7.66 mg dry or 0.5 ml if it was already in solution) were denatured by dissolving in SDS buffer solution (0.5 ml) and incubation for 5 minutes at 95 °C.

Protein standard for gels

A protein standard from BioRad was denatured by diluting 20 times with SDS buffer solution and incubation at 95 °C for 5 minutes. The standard consists of the following proteins: phosphorylase b (97.4 kDA), serumalbumin (66.2 kDA), ovalbumin (45.0 kDA), carbonic anhydrase (31.0 kDA), trypsin inhibitor (21.5 kDA) and lysozyme (14.0 kDA).

Preparation of the SDS gel

Lower Tris (2 ml), acrylamide solution (3.33 ml, 30 %, Roth), water (2.67 ml) and ammonium persulphate solution (40 µl, 10 % (m/v) in water) were mixed. TEMED (N,N,N',N'- tetramethylene diamine, 4 µl) was added just before the gel was transferred to the electrophoresis plate to initiate polymerisation. After transferring the gel to an electrophoresis plate, isopropanol was added onto the gel to ensure a smooth surface for the application of the collection gel. The gel was then left for 30 minutes to complete the polymerisation process. The isopropanol on top of the separation gel was decanted and the gel rinsed with water. Upper Tris (1 ml), acrylamide solution (0.52 ml, 30 %, Roth), water (2.47 ml) and ammonium persulphate solution (40 µl, 10 % (m/v) in water) were mixed. TEMED (4 µl) was added, just before the gel was transferred on top of the separation gel, to initiate polymerisation. The sample application comb was inserted into the application gel and the gel was left for 30 minutes to polymerise.

Electrophoresis

The sample application comb was removed from the gel and the electrophoresis plates were transferred to the electrophoresis chamber and the chamber filled with electrode buffer. The samples and standard (10 µl of each) were applied to the gel and an amperage/circuit of 10 mA was applied to the gel for 10 minutes followed by 25 mA for 50 minutes.

Coomassie staining

Coomassie Brilliant Blue (1 g), acetic acid (100 ml), methanol (300 ml) and water (600 ml) were mixed. The gel was incubated in the staining solution for 30 minutes, whereafter it was destained in a destaining solution comprising methanol (300 ml), acetic acid (100 ml) and

water (600 ml) for another 30 minutes. During the Coomassie staining all the protein bands on the gel are coloured dark purple (Bradford, 1976).

Activity staining

Solution A: 1-Naphtyl acetate (20 mg) was dissolved in acetone (5 ml) and added to Tris buffer (50 ml, 0.1 M, adjusted to pH 7.5 with 1 M hydrochloric acid).
Solution B: Fast Red TR (50 mg) was suspended in Tris buffer (50 ml, 0.1 M, adjusted to pH 7.5 with 1 M hydrochloric acid).

The proteins were renaturated by leaving the gel for 30 minutes to incubate in a Triton solution (0.5% in 0.1 M Tris/HCl pH 7.5). The gel was then incubated for 30 minutes in a 1:1 mixture of solutions A and B. The lipases hydrolyse the 1-naphtyl acetate to naphtol and acetic acid. The naphtol reacts with Fast red TR to form a diazo dye which causes a red stain on the areas of the gel that contains hydrolytically active proteins. The gel is destained by incubation for 30 minutes in a solution of methanol (30 ml), acetic acid (10 ml) and water (60 ml).

Iso-electric focusing

Instrumentation

Pharmacia LKB PhastSystem

Chemicals and Materials

PhastGel pH 5-8 IEF polyacrylamide gels, PhastGel IEF,3-9 pI calibration standards from Pharmacia Biotech.

Sample preparation

Lipase of *Aspergillus niger* from Amano (12 mg) was dissolved in phosphate buffer (pH 7.5, 500 µl) and diluted 2 times. Both the undiluted and diluted enzyme solution as well as a high molecular weight standard (2 µl) were applied to the gels in duplicate. The method in **Table 2** was used.

Table 2: Separation method used in IEF with PhastGel IEF 5-8 gels and the PhastSystem

Sample applicator down at	2.2 0 Vh
Sample applicator up at	2.3 0 Vh
SEP 2.1 2 000 V 2.0 mA	3.5 W 15°C 75 Vh
SEP 2.2 200 V 2.0 mA	3.5 W 15°C 15 Vh
SEP 2.3 2 000 V 5.0 mA	3.5 W 15°C 510 Vh

Silver staining

Silver staining was done according to Butcher and Tomkins, (1986).

Determination of the total protein content of the commercial lipase

The crude enzyme (7.40 mg) supplied by Amano was diluted to 0.267 mg/ml, 0.400 mg/ml and 0.0667 mg/ml with phosphate buffer (0.1 M, pH 7.5). Bicinchoninic acid (BCA)-assay solution (from Pierce, Rockland, USA) (1 ml) was added to these dilutions (50 µl) in triplicate and incubated at 60 °C for 30 minutes. The absorption of these enzyme samples was then measured at 562 nm. BCA-assay solution which consists of two ready-to-use solutions, (A and B) is prepared by mixing 50 aliquots of A with one aliquot of B. The assay solution contains Cu^{2+} and BCA. In the presence of proteins, Cu^{1+} is formed which forms a colour complex with BCA. A standard curve ($y = 0{,}00399x + 0{,}00963$, $R^2 = 0.9996$), was constructed with a bovine serum albumin (BSA) test solution (from Pierce, Rockland, USA)(See **Table 3** for results).

Table 3: Results of the BCA assay of the crude lipase from Amano to determine its protein content

Crude lipase concentration	Absorption	Protein concentration	% Protein content
0.00667 mg/ml	0.060	0.0126 mg/ml	18.9 %
0.267 mg/ml	0.202	0.0482 mg/ml	18.1 %
0.400 mg/ml	0.294	0.0713 mg/ml	17.8 %

RESULTS AND DISCUSSION

The SDS gel of the enzyme showed more than 32 bands (See **Figure 6**). The hydrolytically active proteins have all very similar molecular weights and could only be separated with iso-electric focussing (See **Figure 7** and **8**). The protein content of the lipase was determined to be around 18% (**Table 3**).

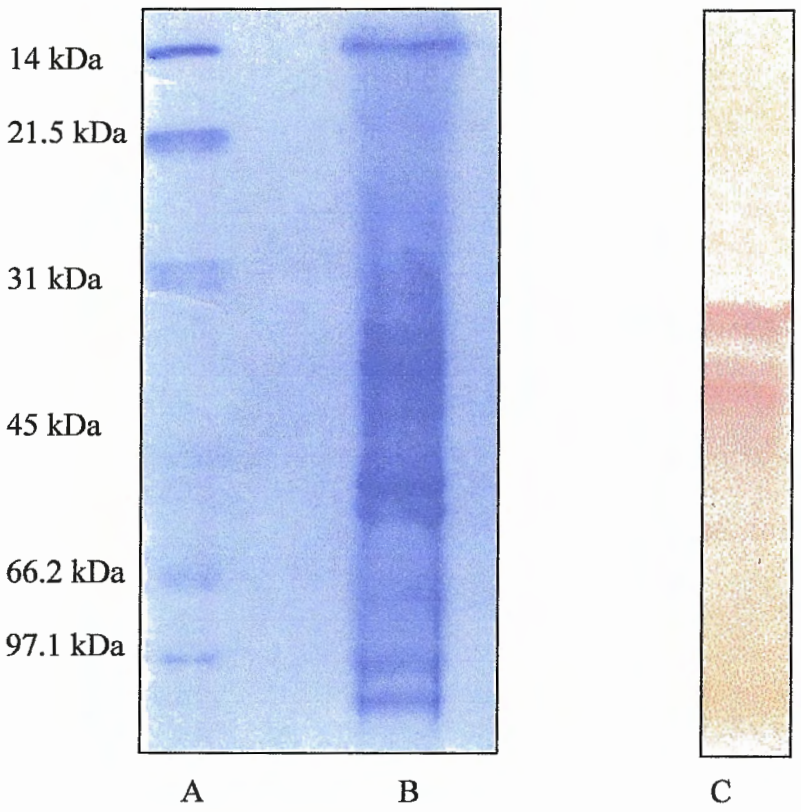


Figure 6: SDS acrylamide gel of a low molecular weight standard (A) and the commercial lipase (B) (Coomassie stained) and SDS acrylamide gel with the commercial lipase (C, Activity stained).

Figure 7: Iso-electrically focussed acrylamide gel (Silver stained): A = standard, B and C = commercial lipase.

Figure 8: Iso-electrically focussed acrylamide gel (Activity stained): A = higher concentration commercial lipase, B = lower concentration commercial lipase

PURIFICATION OF THE LIPASE OF *ASPERGILLUS NIGER* OBTAINED FROM AMANO

Instrumentation

Pharmacia LKB, 2 pump system, equipped with a Pharmacia LKB Controller LCC-501 Plus, optical UV unit and a LKB FRAC 200 fraction collector.

Sample preparation

Lipase (1.98 g) from Amano was dissolved in phosphate buffer (10 ml, pH 7.5) with phenylmethylsulfonyl fluoride (PMSF, 1.7 mg) as protease inhibitor. The lipase solution was dialysed in a Spectra/por molecularporous membrane (MWCO: 6-8 000, Spectrum Medical Industries, California) for 48 hours in distilled water.

Hydrophobic Interaction Chromatography

Method

A glass column was packed with Phenyl Sepharose Fast Flow (180 ml). A starting buffer of 10 mM NaH_2PO_4, 1 M NaCl, pH 8.5 was pumped through the column (120 ml) followed by a gradual increase of the ending buffer, 0.01 M NaH_2PO_4, pH 8.5 until only the pure ending buffer was pumped through the column after 200 ml at a flow rate of 1.5 ml/min.

The elution of hydrolytically active proteins was monitored by a colour reaction with *p*-nitrophenyl acetate: The eluted fractions (15 µl of each) in phosphate buffer (130 µl, 0.05M, pH7.5) were reacted with *p*-nitrophenyl acetate (15 µl, 10 mM) in microtitre plates and the absorbance was measured at 504 nm after 10 minutes at room temperature.

The protein content of the active fractions was measured using a standard Bradford test and the fractions were combined according to their relative hydrolytic activity and protein content.

Results and Discussion

No hydrolytically active fractions were found, even after 100 % isopropanol was pumped though the column. The active enzymes were either denatured or were firmly held on the packing material of the column.

Cation Exchange Chromatography

Method

A glass column was packed with SP Sepharose Fast Flow (200 ml). A starting buffer of 10 mM $CaCl_2$, pH 5.5 was pumped through the column (120 ml) followed by a gradual increase of the ending buffer, 10 mM $CaCl_2$, 1 M NaCl, pH 5.5 until only the pure ending buffer was pumped through the column after 200 ml at a flow rate of 1.5 ml/min. The elution of the hydrolytically active enzymes was monitored in a similar way as in the hydrophobic interaction chromatography.

Results

The cation exchange packing material did not retain the crude enzyme sufficiently and no separation was achieved under the above conditions.

Gel Filtration Chromatography

Method

A glass column (80 x 2 cm) was packed with SP Sephacryl S-200 HR (500 ml). An isocratic elution of 0.05 M NaH_2PO_4, 0.1 M NaCl, pH 7.5 was employed with a flow rate of 0.04 ml/min. The elution of the hydrolytically active enzymes was monitored in a similar way as in the hydrophobic interaction chromatography and all of these were eluted after 20 hours.

Results

Although separation was achieved, it was not sufficient and a further separation step was still necessary (see **Figure 9**).

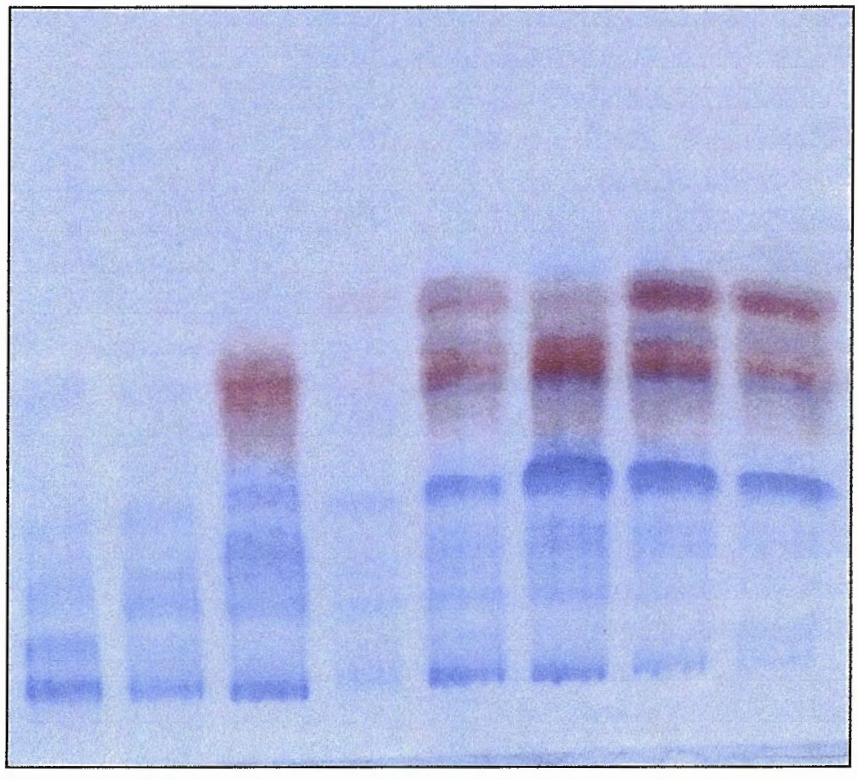


Figure 9: SDS acrylamide gel of the combined active fractions after gel filtration chromatography with the fractions that eluted first on the right (1) followed by the fractions that eluted later (8). The gel was both Coomassie- (blue) and activity (red) stained.

Anion Exchange Chromatography

Method

A glass column was packed with Super Q 650 M (65 µm, 180 ml). A two phase mobile phase was used: A: 20 mM Tris/HCl, pH 8.0 and B: 1 M NaCl, 20 mM Tris/HCl, pH 8.0.

After the column was conditioned with mobile phase A (80 ml), the lipase solution (9 ml) was transferred onto the column. An elution program indicated in **Figure 10** was used and 3 ml fractions of the eluant were collected.

The elution of hydrolytically active proteins was monitored in a similar way as in the case of the hydrophobic interaction chromatography.

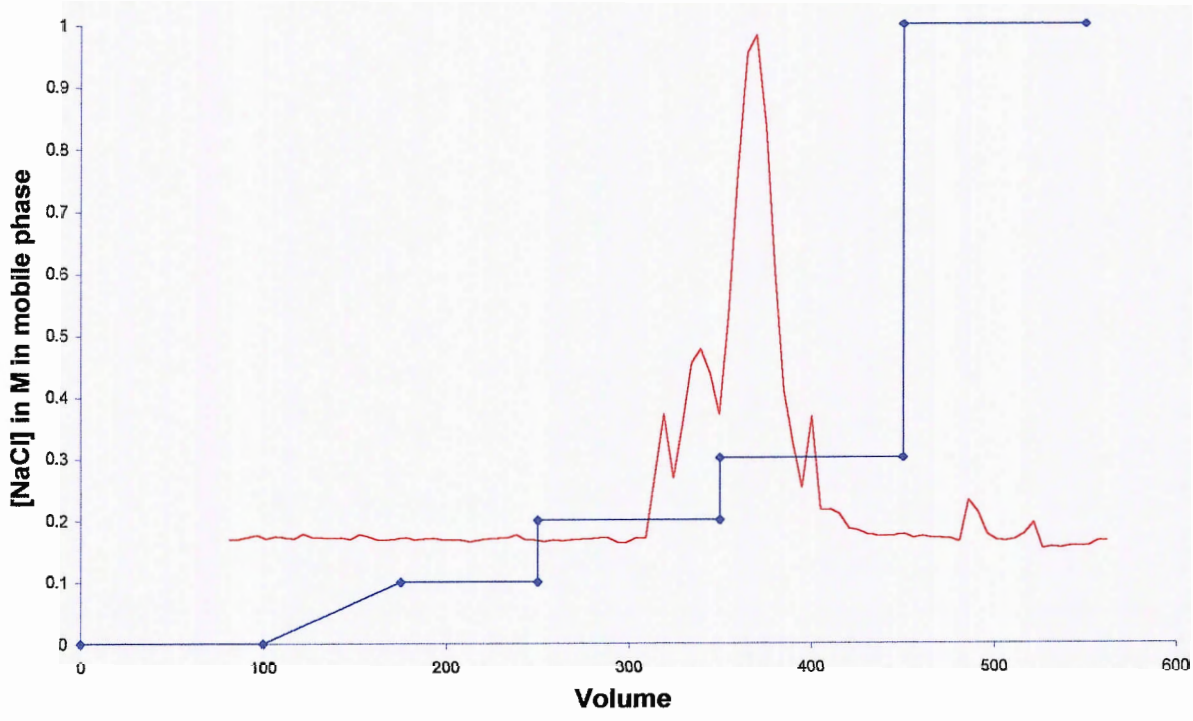
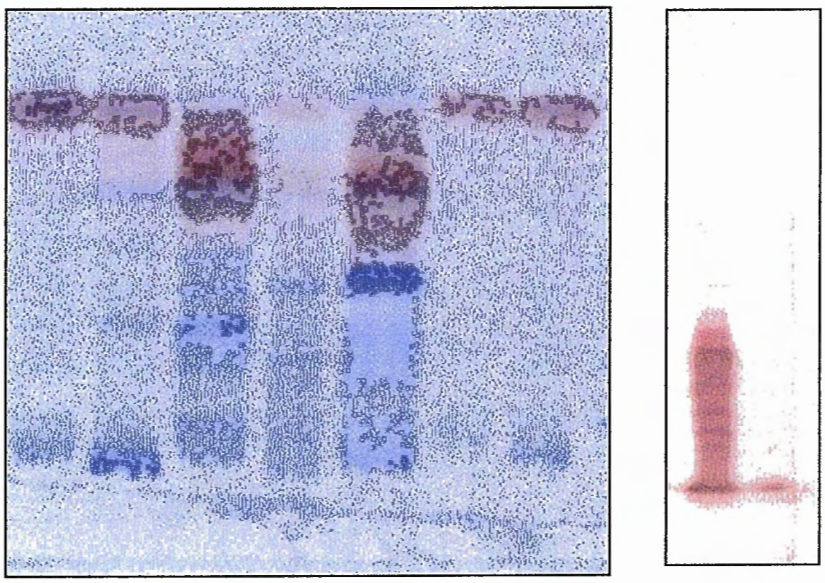


Figure 10: Anion exchange chromatogram of the lipase from *A. niger* showing the elution profile of the hydrolytically active enzymes (red) and the elution program in blue indicating the percentage that solution B constitutes in the mobile phase.

Results

Figure 11 clearly illustrates that the enzyme was purified to an extent: Only one band is visible in the two fractions on the sides of the SDS gel (number 1 and 7). The presence of only one band on the iso-electric focussed gel confirmed that the anion exchange chromatographic method was the best chromatographic method tested to purify the lipase. The pure fractions were combined and blotted on a polyvinylidene difluoride membrane (0.45 µm pore size, obtained from Millipore) (Madsudaira (1987). The band that corresponds to the active enzyme was cut out and the protein sequenced according to the method of Coull *et al.*, (1991). The sequencing yielded the following N-terminal sequences: SESAQQVTDT and VGPL(K)SM(T)LMLL. The results were, however, not completely unambiguous. The purified enzyme was confirmed to be a lipase by the cleavage of *p*-nitrophenyl palmitate and it was also proved to cleave OTA to OTα.



1 2 3 4 5 6 7 8 9

Figure 11: SDS gel (both Coomassie and activity stained, left[1-7]) and IEF gel (only activity stained, right[8,9]) of the different chromatographied fractions of the lipase, with 5 and 8 the unpurified lipase and 1, 6, 7 and 9 the purified enzyme.

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