Rapid Purification and Partial Characterization of an Extracellular Enantioselective Lipase from *Aspergillus niger*

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ABSTRACT

Lipases [EC. 3.1.1.3] are capable of hydrolyzing ester bonds of triglycerides and have received much attention from protein researchers after these enzymes have shown potential applications such us, in the manufacture of commercially important chiral drugs due to their enantioselectivity properties. A novel extracellular lipase produced by *Aspergillus niger* was purified and partially characterized. Using the culture medium as crude extract, the lipase was purified to 25.7 folds after Ultrafiltration and DEAE ion exchange chromatography. The enzyme was characterized to have optimum activity at neutral pH and at 30-35°C, while its kinetic parameters, V_{max} and K_m, were determined to be 1.80 U/mL/min and 0.2 ml, respectively. SDS-PAGE analysis revealed the presence of at least two bands (±87 and ±72 kDa), while native PAGE showed a single band. The two bands could represent subunits in a complex or isoforms of the enzyme. The use of the enzyme in the hydrolysis of a racemic mixture of 2-arylpropionic butyl ester analyzed after HPLC demonstrated an apparent predominant enantioselectivity for the S (+) enantiomer. Thus, this lipase is a promising enzyme for the chiral drug preparation of single enantiomer of 2-arylpropionic acid (ibuprofen).

Keywords: lipase, *Aspergillus niger*, purification, enantioselectivity, chiral drug, ibuprofen

Introduction

The global market for single enantiomer of chiral drugs started to surge in 1994 with 45.2 million dollars and reached 123 million dollars in 2000 (Stinson, 2001). In 2001, this market mounted to 147 million dollar, equivalent to an increase of 10.6 percents from the previous year (Challener, 2002). This fast growing market has stimulated a flurry of activities as drug companies, fine chemical firms and academic research groups explore new ways to make single enantiomer compounds. Among the methods applied in the making of enantiomeric drugs, resolution of racemic mixtures is considered more economical than asymmetric synthesis.

S(+)-ibuprofen is a non-steroidal anti-inflammatory drug (NSAID), which has been introduced since last decade. But until recently, all have been marketed and consumed as racemic mixtures except naproxen and flunoxaprofen. Today, there is an increasing interest to produce the pure enantiomeric form of biologically active drugs to prevent any side effects from using racemic mixture. NSAID is a drug that suppresses inflammation in a manner similar to steroids, but without the side effect of steroids. Ibuprofen, which is commonly used to treat the symptoms of arthritis, gout, bursitis, painful menstruation and headache, is a chiral drug where the (S)-enantiomer is responsible for the desired therapeutic effect. The (R)-enantiomer, on the other hand, can undergo unidirectional metabolic inversion of configuration to the active (S)-enantiomer (Sheldon, 1993).

Lipases [EC. 3.1.1.3] hydrolyze ester bonds of triglycerides, resulting in the release of free fatty acids, monoacylglycerols, diacyl-glycerols and glycerols. During the past decade, microbial lipases have received much attention from protein chemists after these enzymes were shown to remain active in adverse experimental conditions,

e.g. high temperature (Sugihara *et al.*, 1990 and 1991; Oliveira & Cabral, 1993; Ohnishi *et al.*, 1994; Krieger *et al.*, 1997), and in the presence of organic solvents (Nagao *et al.*, 1998). Some were found to be stereo-selective (Erdmann, 1988), a property explored by chemists in kinetic resolution of racemic compounds and in asymmetric synthesis.

Lipase-catalyzed reactions have proven to be interesting and have found useful applications in the manufacture of commercially important chiral drugs like Ibuprofen, Naproxen etc. (Sheldon, 1996). Previous studies have revealed the use of fungal lipases in the preparation of active compounds commonly used for the manufacture of chiral drugs. Carrea *et al.* (1996) using commercial lipase of *Aspergillus niger* revealed its enantioselectivity towards the hydrolysis of 3-O-acetate of cephalosporin C, a well known β-lactam antibiotic. Also, Sanchez *et al.* (2000) investigated the enantioselectivity of *Rhizomucor miehei* lipase on ibuprofen and recently, Hongwei *et al.* (2005) reported the kinetic resolution of ibuprofen using *Candida rugosa* lipase.

Here, we present a study on *A. niger* lipase which has an enantioselectivity property towards the hydrolysis of a racemic arylpropionic butyl ester. These results indicate promising potential of *A. niger* lipase for pharmaceutical application to produce important single enantiomer of chiral drug ibuprofen.

Materials and Methods

Lipase production of *Aspergillus niger*

Aspergillus niger (obtained from the collection of Microbilogy Department of UPLB) previously cultured in 3.9% Potato Dextrose Agar (PDA) slants for 72 h was cultivated in a 1000-ml production medium, each containing 1.0% olive oil, 1.0% sucrose, 0.1% Ammonium nitrate (NH_4NO_3), 0.2% potassium dihydrogen phosphate (KH_2PO_4), 0.4% magnesium sulfate ($MgSO_4$) and 0.1% ferrous sulfate ($FeSO_4$), at pH 5.5 for 96 h at at room temperature with orbital shaking. To maintain the integrity of lipase from protease activity, phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 1 mM to the culture before filtration. The fermentation fluid was then filtered through Whatman #1 filter paper to remove fungal cells. The filtrate containing extracellular lipase was used as the crude enzyme preparation and stored at -20°C until use.

Protein content determination

Direct measurement

Rapid measurement of protein content of supernatant layers from *A. niger* cultures was determined by reading the absorbance at 280 nm.

Bradford method

The Bradford method is a dye binding assay (Bradford, 1976) with Coomassie Brilliant Blue R-250 as reagent. A 100-µl of sample was mixed with 5 ml of Coomassie Brilliant Blue solution. The mixture was allowed to stand for 5 minutes at room temperature to allow the complex to form. The absorbance was later read at 595 nm. Protein standard consisting of bovine serum albumin (BSA) at concentrations ranging from 0.001 to 0.1 mg/ml were prepared. The absorbance reading of the sample was calibrated against those of the standard.

Lipase assay

Quantitative determination of lipase activity followed the protocol of Liu *et al.* (1973). The assay mixture consisted of 5 ml of 0.05 N Tris-HCl buffer, pH 7.0.1 ml of olive oil, 1 ml of culture supernatant or enzyme solution and 5 ml isooctane. The mixture was incubated for 1 h at 37°C with continuous shaking. The enzyme reaction was quenched by adding 20 ml of ethanol:acetone mixture (1:1 v/v) and chilling the flask in an ice bath. Fatty acids liberated during incubation were determined by titrating with standardized NaOH (0.05 N) to pH 10.4. One unit of lipase activity was defined as the activity which released 1 µmole of fatty acid under the above conditions.

Lipase purification

Ultrafiltration

The crude enzyme was ultrafiltered through a 76 mm Millipore membrane with 10,000 molecular weight cut-off, to allow smaller molecules to pass through while the bigger molecules remain in solution. A final rinse with 0.05 M Tris buffer pH 7.2 was carried out to dislodge proteins that might bind onto the membrane.

Ion-exchange chromatography

The ultrafiltrate was applied to a DEAE-cellulose column which had been previously equilibrated with 0.05 M Tris buffer pH 7.2. The column was washed with two column volumes of the same buffer. The bound proteins were subsequently eluted with a linear gradient of salt (NaCl) concentration from 0.1 to 1.0 M. Active fractions were pooled into one fraction and concentrated using ultrafiltration.

Gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was carried out on 10% separating gel containing 10 ml of acrylamide stock solution, 11.2 ml of 4x separating buffer pH 8.8, 11.2 ml of distilled water, 0.3 ml of SDS (this was not present in non-denaturing PAGE), 40 µl of N,N,N',N' - tetramethylene-diamine (TEMED) and 100 µl of 15% ammonium persulfate (APS). The 5% stacking gel contained 2.5 ml of acrylamide stock solution, 5.0 ml of 4X stacking gel buffer pH 6.8, 0.2 ml of 10% SDS, 1.0 ml of 15% APS, 15 µl TEMED, and 11.3 ml of distilled H_2O (Rosenberg, 1996).

Denaturing gel electrophoresis

As much as 100 µl of each sample and 5µl of low molecular weight markers were each incubated for 5 min in boiling water bath with 0.5 volume of 2x sample buffer composed of 4% (w/v) SDS, 12% (v/v) glycerol, 50 mM Tris, 2% mercaptoethanol (v/v) and 0.01% Coomasie Brilliant Blue adjusted to pH 8. About 70 µl of each sample were loaded in each well. The run was calibrated against 5 µl of marker composed of: myosin (212 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferin (76 kDa), and glutamic dehydrogenase (53 kDa). The Buffer system was composed of 0.025 M Tris base, 0.13 M glycine and 0.1% SDS.

Non-denaturing gel electrophoresis

The same procedure (in denaturing gel electrophoresis) was employed for non-denaturing PAGE but with the elimination of sodium dodecyl sulfate (SDS) in the reagents.

Visualization of the bands

Protein bands on the gel were visualized using silver staining method according to Rosenberg (1996). The gel was submerged in large volume of fix solution containing 50% v/v of methanol: 12% v/v of acetic acid for 1 h or overnight. Then, the gel was washed with 50% ethanol 3 times for 20 min each and subsequently soaked in 0.02% sodium thiosulfate solution for exactly 1 min. The gel was then washed with distilled water three times for another 20 min each then immersed in 0.2% $AgNO_3$ solution for 10 min. The gel was then rinsed with water two times for 20 sec each. To visualize the proteins, the gel was incubated at room temperature for 10 min in developing solution (Na_2CO_3 60 g/l, 0.5 ml/l of 37% formaldehyde and 4 mg/l $Na_2S_2O_3.5H_2O$), and then washed with water twice for 2 min each to stop the developing process. The gel was soaked again in fix solution for 10 min and finally washed with 50% methanol for at least 20 min.

Lipase characterization

Effect of temperature on lipase activity

The purified lipase was assayed at different temperatures ranging from 30-60°C to determine the optimum temperature of lipase activity. The procedure followed that of the enzyme assay but the temperature was varied.

Effect of pH on lipase activity

The pH of enzyme assay medium was varied from pH 4 to pH 8 at the optimized temperature to determine optimal pH of activity.

Effect of concentration of substrate on lipase activity

Different concentrations of substrate, that is, olive oil from 100 µl to 1 ml were added to the enzyme media containing fixed amount of enzyme and at optimized conditions of temperature and pH.

Lipase-catalyzed hydrolysis of arylpropionic butyl esters

Hydrolysis of arylpropionic esters

One ml of racemic arylpropionic butyl ester dissolved in 10 ml isooctane was added into 10 ml of 0.05 M phosphate buffer, pH 6.0, in a 250 ml erlenmeyer flask. About 2.5 ml of enzyme solution derived from the active fraction for each step of purification was added into the prepared solution. Subsequently, the mixture was incubated for 72 h at room temperature with continuous shaking. The reaction was stopped by adding 10 ml of acetone:ethanol (1:1 v/v). The organic layer was separated from the aqueous layer using a separatory funnel. The aqueous layer was then

washed twice by using isooctane to remove unreacted butyl ester and acidified with HCl to pH less than 3.0. The aqueous layer was then extracted twice with isooctane to isolate the free arylpropionic acid. Both organic extracts were dried.

HPLC analysis of hydrolysis product

Arylpropionic acid (ibuprofen) obtained during the hydrolysis was analyzed using a Hitachi High Performance Liquid Chromatography (HPLC) D-7000 set with R,R Whelk-O 1 chiral column. The mobile phase used was hexane:isopropanol (98:2 v/v) containing 0.5 ml acetic acid per 100 ml of solution. The operating pressure was set at 270 psi with a flow rate of 0.600 mL/min. The elutes were monitored using an online UV detector set at 254 nm. To confirm the identity of peaks, spiking was done using racemic and S-(+)-aryl propionic acid standard.

Results and Discussion

Lipase production

Lipase from *A. niger* was produced in a production medium after incubation for 96 h, an incubation period identified to produce high yield. The culture was filtered in order to separate fungal cells including mycelia from culture fluid containing lipase.

The lipase production was also optimized by modifying the nutrients of culture media. One percent of sucrose was used as the main carbon source for mass production. This would supply twice as much carbon compared to glucose as reported by Garingan (1998). The more mycelia produced, the more lipase might be obtained. In order to induce more lipase, 1% olive oil was used. Olive oil can be used as subsequent source of carbon after the sucrose is exhausted.

Spectrophotometric analysis showed that the protein content of the filtered crude extract was 0.595 µg/ml while the volumetric activity of lipase was 1.33 U/ml protein. The obtained protein content of the culture was almost twice that of Garingan's (1998) lipase at 0.33 µg/ml. This higher protein content was obtained by changing the nitrogen source from sodium nitrate as previously reported to ammonium nitrate. The latter provided approximately 35% nitrogen needed for protein synthesis while the former only supplied about 17% nitrogen.

Lipase purification

DEAE-cellulose ion exchange chromatography

Table 1 shows the results of enzyme activity analyses done on the fractions eluted from the DEAE-cellulose column. The 0.1 M NaCl fraction had the highest lipase activity (3.42 U/ml) which was used for further analysis. The results suggest this lipase is relatively hydrophobic and binds weakly to the matrix, causing it to elute at low salt concentration. This differs from the result obtained by Garingan (1998) where high concentration of salt (0.7 M NaCl) was needed to elute an S (+) enantioselective lipase.

The chromatographic step was very significant in increasing relative purity of the enzyme based on the purification value. The 0.2 M NaCl fraction showed highest degree of purity; however, it was limited by the reduced yield. Highest yield was demonstrated by the 0.1 M NaCl fraction which ranked second in terms of purification fold and specific activity. Subsequent steps then made use of 0.1 M NaCl fraction.

SDS polyacrylamide gel electrophoresis

To monitor the progress of purification, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out. SDS-PAGE analysis of the crude extract and lipase-positive fraction showed two bands after silver staining (Fig 1). Rf value analysis of protein marker and target protein revealed that molecular weight of the two major bands are approximately 87 kDa and 72 kDa, respectively. Previous reports show that molecular weight of commercial *A. niger* lipase A is 32 kDa (Carrea *et. al.*, 1996). In addition, Garingan (1998) reported that lipases from *A. niger* have 2 main bands with molecular weight 62.9 and 49.6 kDa respectively. The results could indicate that *A. niger* has several lipase isozymes or isoforms which have different substrate recognition capability and specificity.

Non-denaturing polyacrylamide gel electrophoresis

To prove that this lipase might consist of more than one subunit or isozyme, non-denaturing (native) PAGE was carried out. This method allows the quaternary structure of the protein to remain intact and also indicates the state of homogeneity of sample. It can also predict the number of isoforms. It is interesting to state that

Table 1. Lipase activity of crude extract and DEAE-cellulose bound fraction using different concentrations of NaCl solution

Fraction	Protein content (mg/ml)	Volumetric activity (U/ml)	Specific activity (U/mg Protein)	Purification fold	Yield
Crude extract	95.0	1.33	14.0	1.0	100
0.1 M NaCl	9.5	3.42	360	25.7	77.1
0.2 M NaCl	2.3	1.26	547.8	39.1	28.1

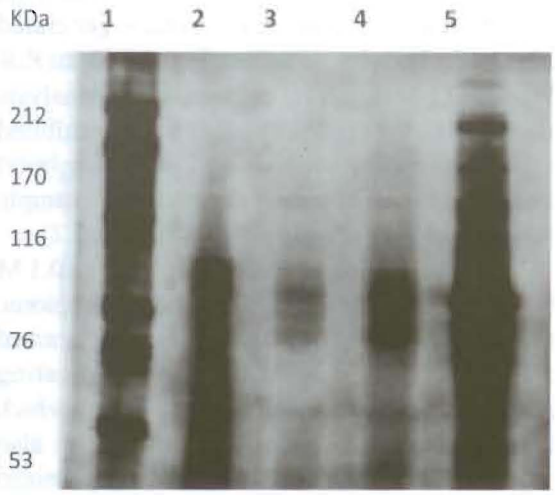

Figure 1. SDS-PAGE of purified lipase from *A. niger* after silver staining. Lane 1, high molecular weight protein marker (Amersham Parmacia Biotech); myosin, 212 kDa; α_2-macroglobulin, 170 kDa; β-galactosidase, 116 kDa; transferin, 76 kDa; glutamic dehydrogenase, 53 kDa. Lane 2, DEAE-cellulose fraction eluted with 0.3 M NaCl. Lane 3, DEAE-cellulose fraction eluted with 0.2 M NaCl. Lane 4, DEAE-cellulose fraction eluted with 0.2 M NaCl. Lane 5, crude extract. Arrows (→) indicate the target protein

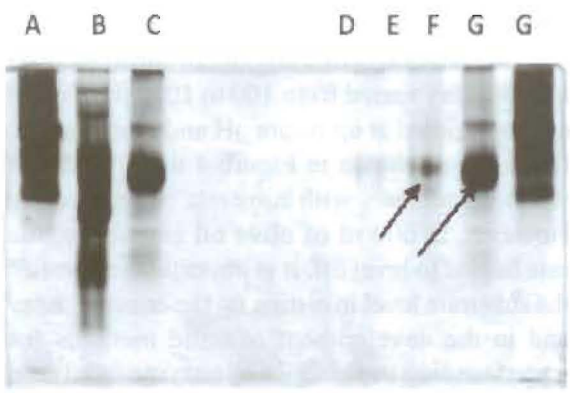

Figure 2. Non-denaturing gel electrophoresis of lipase from *A. niger* after silver staining. Lane A and G, protein marker. Lane B, denatured crude extract. Lane C and F, undenatured crude extract. Lane D, undenatured DEAE-cellulose fractions eluted with 0.2 M NaCl. Lane E, undenatured DEAE-cellulose fractions eluted with 0.1 M NaCl. Arrows (→) indicate the target protein

only one major band appeared after non-denaturing PAGE. This suggests that the lipase from *A. niger*, eluting at 0.1 M NaCl from DEAE column, putatively consists of either two different isozymes with same molecular size or a single enzyme with 2 subunits (Fig 2).

Characterization of purified lipase

Effect of pH on lipase activity

The effect of pH on lipase activity of the purified fractions was determined. Figure 3B shows that the exo-lipase from *A. niger* is active over a wide range of pH but shows optimum activity at pH 7.8.

Holme & Pack (1998) cited that enzymes are very sensitive to changes in pH and work at a defined pH optimum. The effects of pH are due to the changes in the ionic state of the amino acid residues of both the enzyme and the substrate molecules. These alterations in charge will affect substrate binding and the resulting rate of reaction. Extreme change in pH causes permanent denaturation.

Effect of temperature on lipase activity

The increase in temperature increases the rate of chemical reactions, but it also increases the rate of denaturation of protein/enzyme. Determination of the effect of temperature on lipase activity was carried out under the optimum pH by varying the temperature of lipase assay, which ranged from 30-60°C. The activity of lipase exhibited a general decline as the temperature increased. The decline in activity was dramatic

in the range 50-60°C as shown in Fig 3-B. This range of temperature caused the disruption of hydrogen bonding and other forces of molecular interaction in the lipase structure causing the distortion in the conformation, leading to denaturation. Highest activity was obtained in the temperature of 30-35°C. However, the enzyme relatively remained stable as the temperature reached 50°C.

Effect of substrate level on lipase activity

A variation on the amount of substrate used for the assay varied from 100 to 1000 µl of olive oil was studied at optimum pH and temperature. It is clear as shown in Figure 4 that the rate of reaction increases with substrate concentration. However, at 600 µl of olive oil and above, the rate begins to level off. It is important to identify the substrate level in setting up the enzyme assay and in the development of valid methods for understanding the kinetics of enzyme-catalyzed reactions (Holme & Peck, 1998).

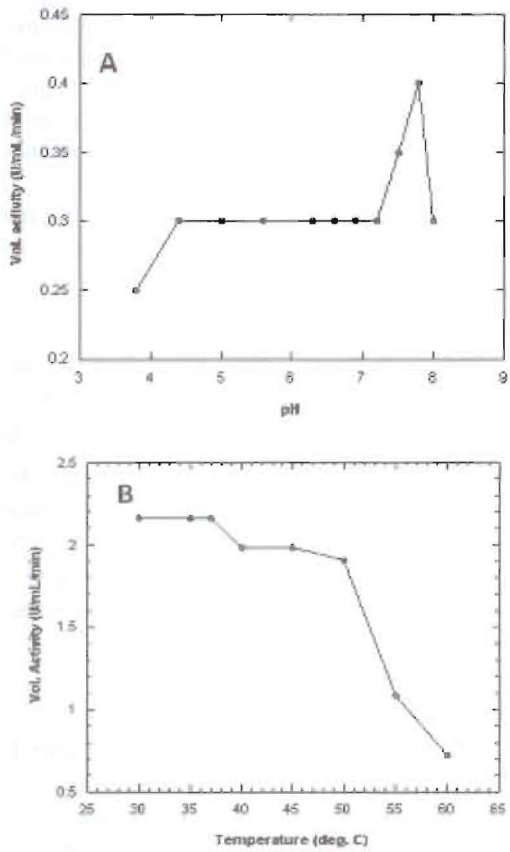

Figure 3. (A) The effect of pH on lipase activity; (B) the effect of temperature on lipase activity at optimum Ph

Lipase-catalyzed hydrolysis of an α-arylpropionic butyl ester

Hydrolysis of an α-arylpropionic butyl ester. The enantioselectivity of *A. niger* lipase was examined in the hydrolysis of butylester of α-arylpropionic acid by HPLC analysis of the hydrolysates using a chiral column.

Product analysis via HPLC

The enantioselectivity of the sample enzyme was evaluated through the products generated after hydrolysis by HPLC equipped with an R,R Whelk-O 1 as the chiral column. HPLC analysis showed that the crude extract of lipase exhibited enantioselectivity towards aryl propionic butyl ester (Fig 5.1). This was validated when sample was spiked with the S(+) Standard (Fig 5.2).

DEAE-Cellulose fraction eluted with 0.1 M NaCl was also able to hydrolyze the arylpropionic acid butyl ester (Fig 6). The hydrolysate contained a predominance of the S(+) acid, indicating preference for the S(+) ester. A small peak which could represent the other enantiomer was also observed. Parameters could be further optimized such as time of incubation to enhance selectivity. Thus, the kinetics of the reaction can still be manipulated to produce more of the S(+) and less of the R(-) enantiomer. A quantitative estimate of unreacted ester was difficult to establish since the work-up did not aim for this only of the acid. This could be gauged accurately the aggressiveness of the enzyme sample.

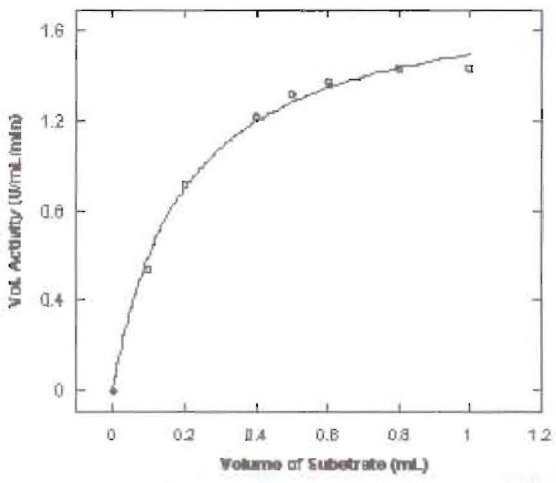

Figure 4. Effect of the amount of substrate on lipase activity at optimum pH and temperature. The amount of enzyme was fixed at 9.5 µg, equivalent to 3.42 U

Figs 5 and 6 suggest that the crude lipase was more selective than purified lipase. The racemic substrate could be in theory, acted upon by both S(+) and R(-) enantioselective enzymes. The difference is in the relative rates of reaction. One of the enzymes could catalyze the hydrolysis at a faster rate. Alternatively, the S(+)-selective enzyme could actually recognize both enantiomers, but with greater efficiency towards the S(+)-enantiomer. If the reaction time is extended, the S(+)-enatiomer concentration becomes limiting, the R(-)-enantiomer then becomes the next substrate. In the experiment, shorter time periods could be studied to establish whether the enantioselectivity of the enzyme is compromised by an extended period of incubation. This might be due to the slight change in the conformation of the catalytic site which recognizes the arylpropionic butyl ester substrate or might be due to absence of cofactor such as magnesium and/or iron which were present in the crude extract. Some enzymes require a cofactor to function well. Garingan (1998) identified a lipase from *A. niger* that was selective for the S (+) enantiomer, this time using an arylpropionic methyl ester as the substrate. This is different from the lipase being assayed since this eluted at high salt concentration using the same column, suggesting a reduced degree of hydrophobicity.

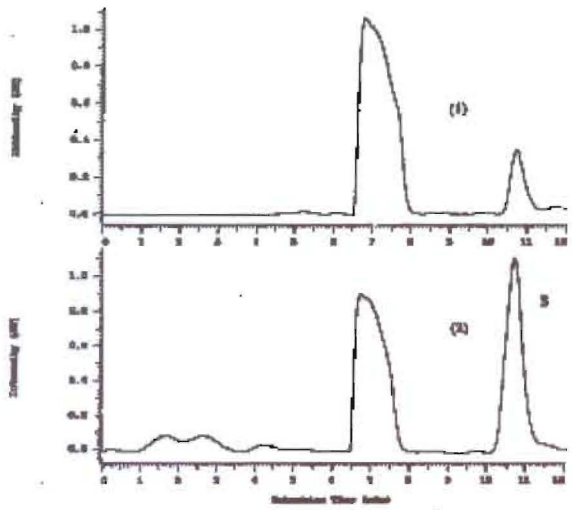

Figure 5. HPLC chromatogram of the isooctane extract of the aqueous phase from the hydrolysis of an arylpropionic butyl ester; 1. Using lipase crude extract; 2. Spiked with the S(+)-arylpropionic acid

These results demonstrate the potential use of *A. niger* lipase in the production of single enantiomer arylpropionic acids. Further step must be taken to evaluate and determine the possible contributions of lipase in this undertaking.

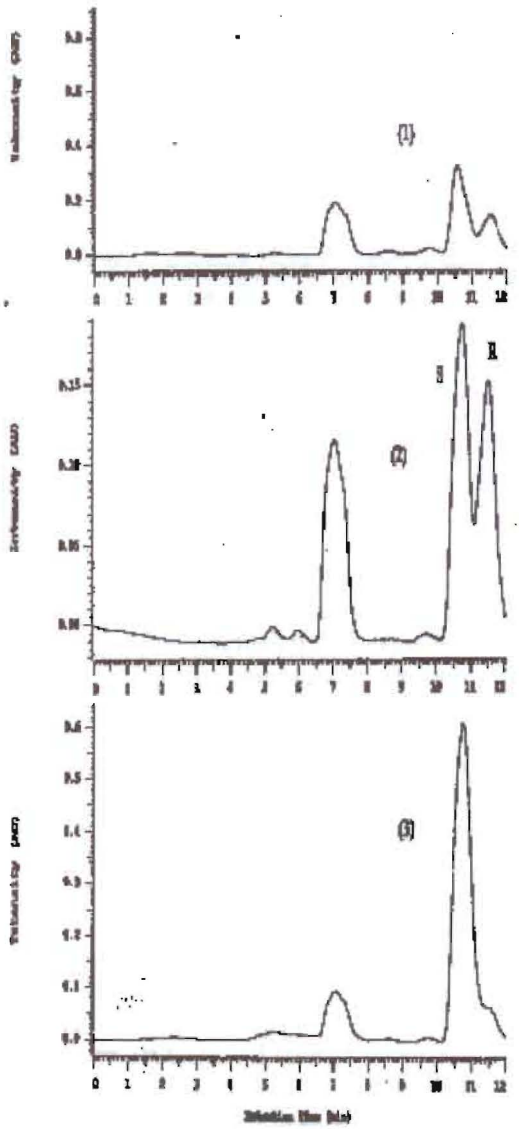

Figure 6. HPLC chromatogram of the isooctane extract of the aqueous phase from the hydrolysis of an arylpropionic butyl ester; 1. Using lipase from DEAE-cellulose fraction eluted with 0.1 M NaCl; 2. Spiked with racemic arylpropionic acid; 3. Spiked with the S (+)-arylpropionic acid

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