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Partial Purification of Intestinal Triglyceride Lipase from *Cyprinion macrostomus* Heckel, 1843 and Effect of pH on Enzyme Activity*

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Abstract: Intestinal triglyceride lipase (TG) of *Cyprinion macrostomus* after precipitation with 30% polyethylene glycol (PEG), was partially purified by hydrophobic interaction chromatography on phenyl sepharose CL-4B, and the effect of pH on this enzyme activity was determined titrimetrically. Intestinal tissue homogenates were precipitated using 30% PEG-8000, and then applied onto a phenyl sepharose CL-4B column for hy-drophobic interaction chromatography. The lipase that bound to this hydrophobic resin and was partially purified by this single step application was then eluted from this resin with taurocholic acid and Triton X-100 elution. This purified enzyme effectively hydrolysed natural olive oil substrate under the experimental conditions. An optimal pH of 7.50, and 94.37% yield, and 71.54-fold purification parameters were estimated for this lipase. The molecular mass of this enzyme was determined to be 51 kDa under non-denaturing conditions by polyacrylamide disc-gel electrophoresis (Disc-PAGE). It was concluded that the intestinal lipase of this fish is alkaline in character.

Key Words: Lipase, purification, enzyme activity, optimal pH.

Cyprinion macrostomus HECKEL, 1843 İncebağırsak Lipaz'ının Kısmi Saflaştırılması ve pH'nın Enzim Aktivitesi Üzerine Etkisi

Özet: *Cyprinion macrostomus* incebağırsak Lipaz'ının% 30 polietilen glikol (PEG) ile çöktürülmesinden sonra, hidrofobik etkileşim kromatografisi ile kısmi saflaştırılması ve pH'nın enzimatik aktivite üzerine etkisi titrasyon yöntemiyle araştırılmıştır. İncebağırsak doku homojenatı %30 PEG-8000 ile çöktürülmüş ve fenil sefaroz ile hidrofobik etkileşim kromatografisine uygulanmıştır. Matriks ile bağ kurmuş olan ve bu şekilde kısmi olarak saflaştırılan lipaz aktivitesi taurokolik asit ve triton X-100 çözeltileri ile geri alınmıştır. Saflaştırılan enzim deneysel koşullar altında doğal zeytin yağı substrat'ını etkili şekilde hidrolizleşmiştir. İncebağırsak lipaz aktivitesi için optimum pH'nın 7.50 olduğu saptanmıştır. %94.37'lik bir verim ve 71.54-kat saflaştırma parametreleri saptanmıştır. Poliakrilamid disc-continuos jel elektroforezi (Disk-PAGE) ile denatüre edici etken içermeyen koşullar altında bu Lipaz'ın molekül kütlesi 51 kDa. olarak tespit edilmiştir. Bu balığın incebağırsak doku lipaz'ının alkali karakterde olduğu sonucuna varılmıştır.

Anahtar Sözcükler: Lipaz, saflaştırma, enzimatik aktivite, optimal pH.

Introduction

Lipases (triglyceride acyl hydrolase, EC 3.1.1.3) are enzymes widely distributed among animals, plants and microorganisms that catalyse the hydrolysis of glycerol ester bonds at the fat-water interface. The isolation and purification of lipases from different sources, mainly microorganisms (1–9) and mammalian tissues, have been reported (10-38). Although it is known that lipase activity exists in several plant tissues, very few studies have been performed on the distribution of lipases in whole plants, except in seeds and fruits (39) and in insects (40). However, in general, there is also a paucity of information about the enzymology of digestive lipolysis in fish (41–43), and therefore the lipases from the tissues of these aquatic vertebrates have received much less attention than those from mammals and microorganisms (44-53). Indeed, there is only limited research about mammalian type pancreatic lipase, which has mainly been

* This research was conducted in 1994 as an MSc thesis and this paper consists of a part of this thesis.

isolated from rainbow trout (*Oncorhynchus mykiss*) pancreas, and the enzymatic properties of the lipase from other tissues has not yet been investigated from other fish species (51-53). On the other hand, as described above, with a few exceptions, studies on fish lipases have been limited to crude extracts (48, 54, 55). Consequently, the possible participation of several enzymes in the observed activity cannot be excluded. For this reason, the isolation and purification of enzymes is necessary in order to study individual enzymes in lipid digestion. This paper focuses upon partial purification and elucidates some biochemical properties of the enzyme responsible for its activity. Therefore, we chose *Cyprinion macrostomus*, which lives in the stream joined to Kangal Hot Spring, in order to evaluate and provide some approximation for cytosolic intestinal lipase level and to determine some chromatographic properties including aspects of partial purification, hydrophobic character and some biochemical properties.

Materials and Methods

Animals

Adult *Cyprinion macrostomus* were obtained from Kangal Topardıç (Sivas) Hot Spring stream and maintained in a container. Then they were taken to the Biology laboratory, and kept in an air circulated water tank for a few days until use. All the samples were caught during the summer (between June and July). Forty-one fish were used in the experiment. Mean length and body weight were 15.94 ± 0.18 cm, and 42.07 ± 2.13 g per fish, respectively. The ambient temperature of the stream was 35 °C.

Tissue Extraction

Immediately after the fish were decapitated, the intestines were dissected and washed with 0.9% potassium chloride (KCl) saline solution, and then homogenisation replaced in 50 mM Tris-HCl buffer solution (pH 7.50) using a B. Braun homogeniser. The crude extract was centrifuged three times (Beckman J2-21 type cooling centrifuge) at approximately 25 000 g until the homogenate clarified. During every centrifugation step, floating cake was removed after the homogenate was sieved on glass wool. After centrifugation, the precipitant was discarded and the supernatant was subjected to polyethylene glycol (PEG) precipitation.

Polyethylene Glycol Precipitation

All the steps (precipitation and purification) were carried out on ice or at 4 °C. The supernatant was subjected to PEG 8000 precipitation (56). For this purpose, solid PEG 8000 was slowly added, with constant stirring, to a first concentration step of 20% (w/v). After 1 hour of stirring, the suspension was left at + 4 °C, and subsequently centrifuged at 22 500 g. The supernatant was recorded as SI and precipitant PI. In the final step, to the supernatant (SI) necessary solid PEG was added in the similar way to a final concentration of 30% (w/v) and centrifuged following the protocol described above. Precipitant and supernatant was marked as described before (PII and SII). The pH of the new solution was calibrated to 7.50 in every step of the precipitation. The suspension was stored at –20 °C until used; under this circumstance the enzyme activity was stable at least for a few months.

Partial Purification of Lipase

The supernatant solution II (S II) was subjected to phenyl-sepharose CL-4B column for hydrophobic interaction chromatography. For this purpose, 30 ml of solution (containing ca. 115 mg protein) was diluted 5 times by adding suitable buffer (50 M Tris-HCl, pH 7.50), and then was loaded onto a chromatography column (3 x 12 cm) containing 20 ml of phenyl-sepharose that was first equilibrated with 50 mM Tris/HCl buffer, pH 7.50. Then the column was first washed with a 3-column volume of the same buffer, and unbound protein from the column was removed by this washing. The bound protein was eluted by washing 1 % taurocholic acid and 1 % Triton X-100 prepared in 50 mM Tris/HCl buffer solution (pH 7.50), respectively. The active fraction was recovered by washing the column with 3-column volumes of these solutions. Finally the column developed 1 M 3 column volume KCl, prepared in 50 mM Tris/HCl buffer solution. Fractions of 5 ml were collected, and aliquots of 1 ml were assayed for TG-lipase activities as indicated in the Material and Methods. In every step, fractions containing significant lipase activity were used to establish the purification scheme and electrophoresis. In most cases, the fraction containing active protein was concentrated after dry inert matrix (sephadex G-25) was added to the protein solution and allowed to absorb water and other small molecules.

Assay of Lipase

TG-lipase activity was determined by titrimetrically according to Vadehra and Harmon (1) with little modification (57). Lipase assay was performed with an olive oil substrate consisting of 5 ml of 10% olive oil emulsion, 2 ml of 0.015% sodium taurocholate, 2 ml of 3 M NaCl, 1 ml of 0.075% $CaCl_2$, and 5 ml of water. Olive oil containing 2% polyvinyl alcohol was first emulsified by using a Beckman ultrasonic sonicator. The enzyme–substrate mixture was incubated at 37 °C for 10 min and activity, in other words fatty acid liberation, was terminated by adding 10 ml acetone/alcohol (1/1) to the mixture. The liberated fatty acids were titrated with 0.02 M NaOH after added 2-3 drops of phenolphthalein indicator. In general, a stable reaction rate was reached after 10 min and the volume delivered per min and per ml was recorded (Vs). One unit of activity was defined as the amount of enzyme necessary to liberate 1 µmole of fatty acid produced per min for per ml. Activity was calculated as follows (58). For the blank rate determination, titration was run until the delivery rate was constant, and the volume delivered per minute was recorded (10^3 is factor for µmole, D is dilution, 15 is total as-say media, 0.02 molar concentration of NaOH used for neutralization).

$$U/ml = (Vs - Vb) \times 0.02 \times 10^3 \times D \times 15$$

Determination of Optimal pH

The optimal pH of the enzyme was determined in every step of PEG precipitation and after the enzyme was eluted from the column. The pH activity was tested between 4.00 and 10.00 using with a Hanna HI 8314 Membrane Type pH meter. 50 mM Tris-HCl was chosen as the buffer, in order to prevent ionic strength and drastic changes in pH. 1-ml enzyme solution was incubated for 10 min, and then activity was terminated as indicated in the assay procedure.

Determination of Protein Concentration

Protein concentration was determined by the method of Lowry et al. (59) in every step of purification with the use of bovine serum albumin (BSA) as the standard. Firstly, necessary reagent was added to 0.5 ml of sample containing up to 0.5 mg of protein, as described in the method. The solution was mixed well and left to stand for 20-30 min. Then absorbance was measured at 750 nm against a blank of 0.5 ml of sample buffer. Sometimes, since high concentrations of detergent and bile salts caused different colour development, to minimise their deleterious effects protein solutions were diluted out.

Polyacrylamide Gel Electrophoresis (PAGE)

In order to determine the molecular mass of native lipase, native polyacrylamide disc-gel electrophoresis vertical systems (Desaphor VC System for Circulator Gel Electrophoresis, DESAGA Heidelberg, Germany) was carried out using the method of Parish and Marchalonis (60) using 2.7% and 7.7% polyacrylamide for the stacking and resolving gels, respectively. Since the above gel system was commonly used for lipase molecular mass determination, we decided to use this percentage. Proteins were stained with Coomassie brilliant blue G250 (1.5%, w/v) in methanol-acetic acid 45.4: 4.6. Each gel destained in glacial acetic acid-methanol-water 7.5: 5.0: 87.5. BSA (67 kDa), egg albumin (45 kDa), pepsin (35 kDa), and casein (24 kDa) were used for molecular markers.

Chemicals and Abbreviations

Bis (2-hydroxyethyl) amino-tris (hydroxymethyl) methane hydrochloric acid (Tris-HCl), bovine serum albumin (BSA), pepsin, olive oil, sodium chloride (NaCl), calcium chloride ($CaCl_2$), potassium chloride (KCl) and phenyl sepharose CL-4B were purchased from Sigma. Polyethylene glycol 8000 (PEG), Triton X-100, casein, egg albumin, acetone and alcohol were purchased from Merck. (HIC = Hydrophobic interaction chromatography, Disc-PAGE=Discontinuous polyacrylamide gel electrophoresis). The chemicals used in the protein determination and PAGE were of analytical grade.

Statistical Analyses

The statistical analysis of the effect of pH on activity was evaluated by analysis of variance (ANOVA) and comparisons between means were performed with Duncan's Multiple Range test (61). Differences between means were reported as significant if $P < 0.05$. Results are expressed as mean ± SD for the three experiments.

Results

The partial purification procedure is summarised in Table 1. The enzyme was purified 71.54-fold in a yield of 94.37%. The majority of the active protein was located in the upper phase of 30% PEG precipitation. The pellet activity was found to be lower than upper one. The upper phase containing active fraction was applied onto a hydro-

phobic chromatograph with phenyl sepharose CL-4B, and lipase, being bound to this resin, was then eluted by 1% Triton and bile salt wash, respectively from the column. Since the higher concentration was chosen, displacement of bound protein from the matrix (that is Triton and bile salt), and therefore active protein (containing active lipase) was easily recovered from the column. The disc-PAGE electrophoresis run under non-denatured conditions gave only a single large band.

We studied the effect of pH on activity at different stages of purification. Optimal pH activity was found to be in the pH range 5.50 to 8.50, whereas activity was maximum at pH 7.50 in every purification step as seen in Table 2 and Figure 1. After enzyme was eluted from the column active fraction, was used for pH assay. The pH assays for both fractions were similar for optimal pH, whereas the activity range for Triton was found to be larger than for the taurocholic acid fraction. There was hardly any difference between the acid and alkaline pH side on the activity assays for both fractions. Nevertheless, as seen in the same figure (Figure 1), the profile for optimal pH was found to be higher than for the taurocholic fraction for Triton X-100.

Hydrophobic interaction chromatography of a representative 30% PEG is shown in Figure 2. Although

Table 2. Summary of pH effects on partially purified triacylglycerol lipase. Lipase activity was determined on aliquots of the Triton X-100 and taurocholic acid washing fraction incubated for 10 min as described in the Materials and Methods at different pHs as indicated in the table. Tris buffer (50 mM) was used for pH determination. Duncan's Multiple Range test assessed statistical significance. Results expressed as mean ± SD for three experiments. Means with the same letter in each row do not significantly differ at 0.05 level.

pH	1% Triton X-100 fraction Specific activity µmol/mg protein	1% Taurocholic acid fraction Specific activity µmol/mg protein
4.00	140.68 ± 3.72 f	118 65 ± 5.41 g
4.50	168.41 ± 1.43 ef	148.23 ± 3.28 f
5.00	187.48 ± 1.60 de	182.53 ± 0.22 e
5.50	215.37 ± 4.35 cd	204.53 ± 4.44 d
6.00	253.93 ± 1.74 bc	235.59 ± 2.78 c
6.50	267.10 ± 4.77 b	239.23 ± 3.12 c
7.00	301.58 ± 0.67 ab	271.12 ± 0.90 b
7.50	314.13 ± 5.01 a	291.95 ± 4.06 a
8.00	299.99 ± 1.34 ab	283.05 ± 3.95 a
8.50	287.57 ± 1.99 ab	265.27 ± 3.75 b
9.00	265.60 ± 2.28 b	235.66 ± 4.89 c
9.50	239.05 ± 1.42 bc	209.13 ± 4.52 d
10.00	210.92 ± 1.51cd	180.86 ± 5.13 e

Table 1. Summary of partial purification of triglyceride lipase from fish intestine

Fractions	Total* Activity (µmol)	Total** Protein (mg)	Specific Activity (Units/mg protein)	Yield (%)	Purification (Fold)
Crude extract	7800.00	2200.25	3.55 ± 0.09	100	1.00
Precipitant-P1 (10% PEG)	477.00	98.05	4.51 ± 0.05	6.11	1.35
Supernatant-S1 (10% PEG)	7638.00	169.80	45.54 ± 0.33	97.92	13.42
Precipitant-P2 (30% PEG)	487.00	41.00	11.60 ± 0.26	6.24	2.03
Supernatant-S2 (30% PEG)	8296.00	115.60	71.76 ± 0.68	107.64	21.42
Phenyl Sepharose CL-4B Chromatography					
1% Taurocholic acid	7208.00	30.60	235.59 ± 2.78	92.41	66.36
1% Triton X-100	7361.00	28.56	253.98 ± 1.74	94.37	71.54
1 M KCl	.....	34.68	.....	.....	.....

* A Triglyceride lipase activity was determined titrimetrically according to Vadehra and Harmon with of little modification.

** Protein estimation by the method of Lowry et al.

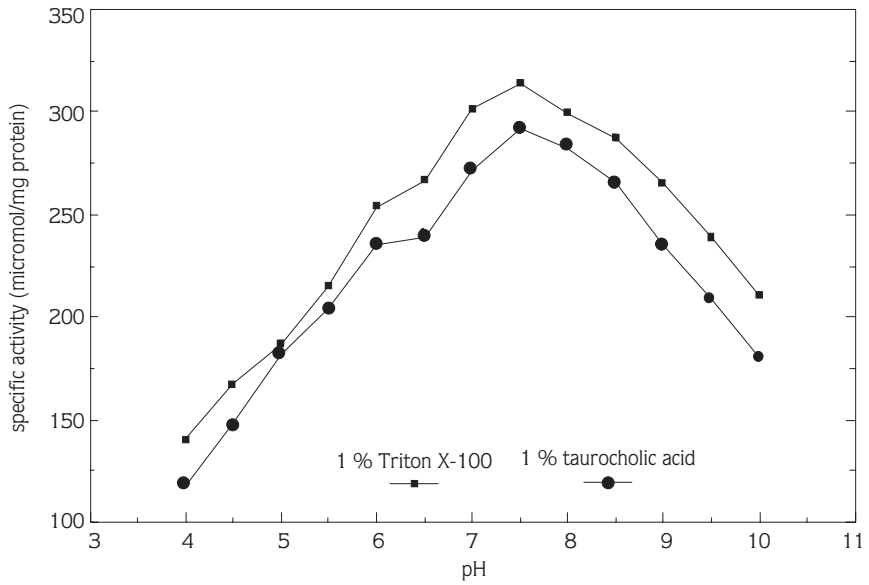

Figure 1. pH profile of partially purified lipase. The activity was measured titrimetrically on olive oil emulsion containing the same medium as indicated in the Materials and Methods (only bile salt is not included since each enzyme solution already contained bile salt -for bile salt elution, and surface active compound –for Triton X-100 elution).

a large number of protein peaks were observed, the majority of the lipase activity was associated with the large fraction, and activity appeared in fractions 33-41 and 42-57 eluted by 1% bile salt and 1% Triton X-100, respectively. As it was explained before, the PAGE electrophoresis run under non-denatured conditions gave

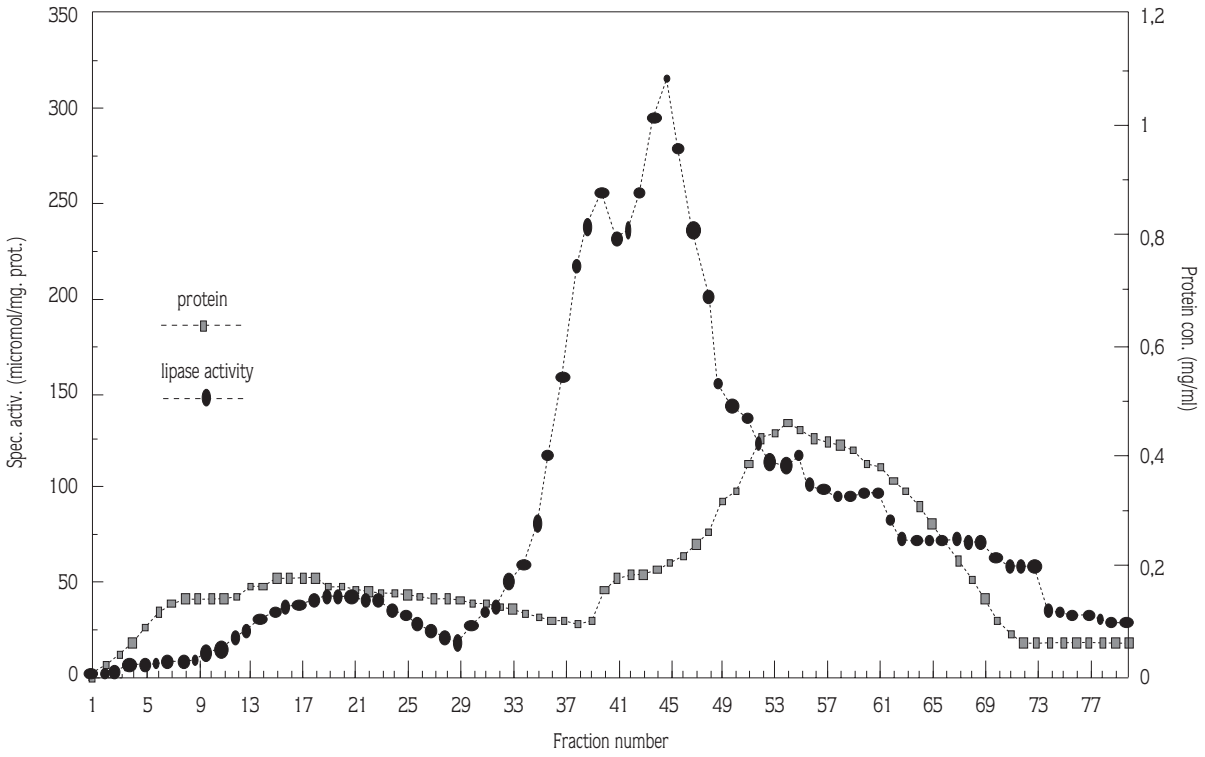

Figure 2. Chromatography of the suspended 30% PEG supernatant fraction on Phenyl Sepharose CL-4B. The column first was eluted (8 ml/h) with a 50 mM Tris-HCl buffer (pH 7.50) solution, and then bound protein was removed from the column by washing with 1% taurocholic-acid and Triton X-100, respectively, prepared in the same buffer. Lipase activities in the effluent fractions (5 ml) were determined as described in the Materials and Methods.

only a major single large protein band corresponding to a molecular mass of ca. 51 kDa for both fractions The logarithmic plot of molecular mass versus mobility in the native disc-PAGE system for the indicated standard and sample proteins and therefore the electrophoretic patterns are shown in Figure 3. As seen in Figure 4, the protein bandwidth for bile salt fraction was much larger than for Triton X-100. Moreover, molecular mass was determined with regard to Triton washing.

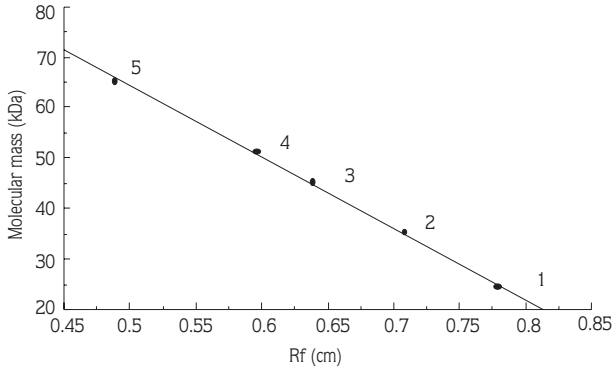

Figure 3. Determination of the molecular mass of *C. macrostomus* intestinal lipase. 25 µL (30µg protein ca.) Sample and known molecular marker applied to the disc-continuous PAGE vertical column. (1: Tripsin inhibitor 25 000, 2: Pepsin 35 000, 3: Egg albumin 45 000, 4: *C. macrostomus* intestinal lipase 51 000, 5: Bovine serum albumin 67 000 Dalton). The logarithmic plots of molecular mass versus mobility in the native disc-PAGE system for the indicated standard proteins were obtained.

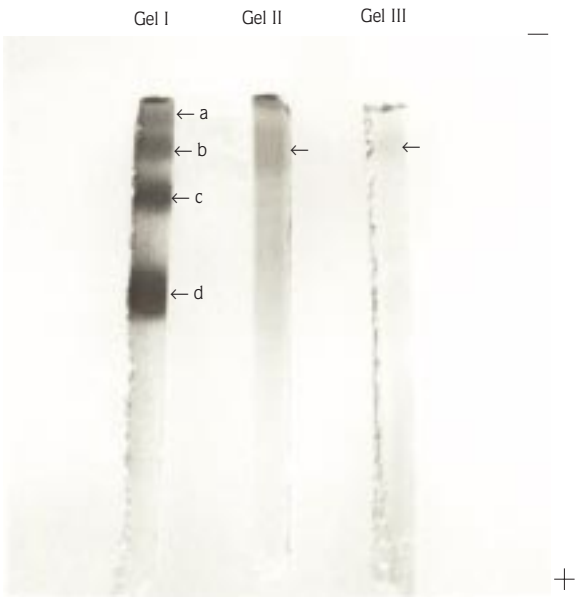

Figure 4. Horizontal arrows indicated sample and standard proteins. Fractions 33-41 (gel II) and 42-57 (gel III) from Phenyl Sepharose column were eluted by 1% bile salt and Triton X-100, respectively. Then each fraction was concentrated directly by adding sephadex G-25 to protein solution, and these solutions were subjected to polyacrylamide gel electrophoresis run under non-denaturing conditions. Protein was stained and destained by the indicated method. The protein migrated as a large single band of 51,000. This was calculated by plotting the Rf values of known proteins against their molecular masses as a logarithmic plot. Molecular mass standards (gel I) were run together in the same vertical column. These standard proteins were bovine serum albumin (a - 67 000), egg albumin (b - 45 000), pepsin (c - 35 000), and tripsin inhibitor (d - 13 000).

Discussion

This work reports the partial purification of a triglyceride lipase from fish intestine and some properties of the enzyme. This partially purified enzyme effectively hydrolysed natural triacylglycerol substrate (olive oil) under specified conditions and gave a large single band in non-denaturing PAGE electrophoresis.

In order to determine a simple purification procedure for partially purifying fish intestinal lipase, we first attempted precipitation of lipase protein using polyethylene glycol–8000 (PEG) and applied the final supernatant (SII) onto a phenyl sepharose CL-4B column for the HIC. This purification step is almost the first attempt so far, and can also be described as a preliminary purification step because washing solution has only been used in the final (high) concentration. In general, before HIC, the adsorption step often requires the presence of salting-out ions such as sodium chloride or ammonium sulphate precipitation, and is commonly recommended in order to obtain a higher yield or recovery (62,63).

One logical assumption about the binding of lipase to this hydrophobic resin for this present purification is that since PEG decreases the availability of water molecules in solution as salting–out ions, then the increase the surface tension might enhance hydrophobic interaction (63). This hydrophobic resin possibly bound this enzyme very tightly. Because we used a high concentration of bile salts followed by a high detergent concentration, the enzyme was easily eluted from the column.

The presence of one major protein band on the non-denaturing PAGE further suggests that the enzyme is in pure form on the gel. Since hydrophobic chromatography is considered a semi-affinity method (sometimes affinity) in some analysis (64), the purification step applied in the

present study is possibly enough for isolation of this intestinal lipase (in general for all lipases) at least in a partial manner.

The hepatic TGL of rainbow trout has also been isolated and partially purified (51) using a similar purification step (purification was achieved after Sepharose Bio–gel A 0.5 m, 200–400 mesh chromatography of a re-suspended 20% ammonium sulphate fraction). In our previous preliminary study, lipase from fish liver was purified by the combinations of ammonium sulphate fraction followed by hydrophobic interaction chromatography on phenyl sepharose (65). Lipase purification from fish liver conducted by us on the same fish liver (*C. macrostomus*) also showed that this enzyme can purify partially using this sort of simple purification technique (57). In that study, similar results such as chromatographic properties and pH characterisation were obtained from the same fish liver (57). Our results suggest that, under the conditions of the present experiment as seen in HIC, hydrophobic forces may be more important in stabilising protein structure. Contrary to what has been found with other enzyme systems, e.g. microbial (8) or mammalian (36,37), the results indicate that other fish lipases such as cod (*Gadus morhua*) show considerably less stabilisation by hydrophobic forces (52,53), but this suggestion is considered controversial, and as yet it is too early to tell whether fish lipases are stabilised less or more by hydrophobic forces, and is still necessary to provide a fuller molecular characterisation of the enzymes (53). The other possible assumption, which was postulated by Takahashi et al., (1988) (66) is that enzymes such as lipase after reacting with an amphipathic molecule (e.g. polyethylene glycol, PEG) which is considered modified, this molecule bound to the surface of the enzyme. Subsequently, the hydrophilic nature of PEG allows the modification of enzyme (lipase) not only in aqueous solution, but also its hydrophobic nature enables modified enzymes to interact with a hydrophobic resin (phenyl sepharose).

Since infranatant was not subjected to ultracentrifugation at 100 000 g (or over), we are not able to decide whether lipase enzyme is cytosolic or not (in those days, it was not possible to provide high speed ultracentrifuge to obtain a cytosolic fraction). Despite the fact that we used 30% PEG in order to precipitate lipase protein, we were not able to precipitate the whole protein in the pellet. As seen in Table 1, majority of the active fraction was observed in supernatant (S II). Because the active protein was located on the upper phase of 30% PEG precipitation, it may be concluded that this enzyme protein a is high-solubility protein rather than a low-solubility one, and must have a cytosolic location in the cytoplasm of the cell (45,48,51,62).

The other results that we have observed in the present study show that 30% PEG precipitation does not protect the binding capacity of the enzyme to this hydrophobic resin. PEG precipitation has been used with considerable success for intrinsically low-solubility proteins, such as globulin. To precipitate for other highly-soluble proteins, it may be necessary to use a higher percentage of PEG or sometimes a higher molecular form of the organic polymers such as PEG. Using the higher molecular form of PEG is limited to some degree since phase separation may occur, with a protein-rich heavy phase separating from a lighter phase above. Nevertheless, a residual low level of PEG is not detrimental and many procedures such as affinity chromatography or gel filtration can be carried out without having to remove the PEG first (62,67).

During the elution step, a high concentration of Tris-HCl was used instead of a lower one to prevent inhibition caused by a high concentration of non-ionic detergent (Triton X-100) and bile salts. Active lipase displacement occurred after bile salt and Triton X-100 washing, respectively. Based on the present results, the active fraction containing lipase activity was first displaced from the matrix by bile salt and then this elution was followed by Triton. In other words, no discontinuity in active lipase elution was observed. The small fragmentation that occurred during the elution profile between bile salt and Triton X-100 (see Figure 2) is not considered to have originated from the solution used in the experiment; instead it might have been caused by technical difficulties in the manual applications. In most studies Triton X-100 is effectively used for the displacement of bound protein (i.e. membrane-bound) from the column. Non-ionic detergents such as Triton X-100 are known to be mild in their action, and most proteins, whether originally membrane-bound or not, can tolerate Triton at levels of 1-3% w/v (62). In addition, 0.5% of Triton X-100 has been used in the purification of insect body lipase, and severe inhibition of this lipase activity caused by a concentration greater than this has been reversed by diluting the detergent (15,40).

The addition of Triton X-100 to human plasma lipoprotein lipase up to 0.5% leading to stabilisation of this enzyme activity during the purification procedure has also been reported (15). However, as the present assay was run under identical conditions, it can be expected that severe inhibition might have occurred in the lipase activity and therefore, to some degree, the purification parameter may be low. Nevertheless, one aim of the present study was only to purify (at least partially) the active lipase protein; the inhibition caused by detergent or bile salts over lipase activity was completely ignored. However, it should be kept in mind that, in subsequent studies, additive compounds such as detergent or bile salts after the enzyme is eluted from the column must be eliminated from the solution of protein with the aid of available methods (mineral precipitation or dialysis) (62,63), or the solution can be diluted to a suitable volume (15,40).

The intestinal enzyme was active in a broad pH-range, especially on the lower pH side. However, the pH activities profile for Triton was higher than for bile salt elution (Figure 1). As mentioned before, this could have originated from the potential activator behaviour of detergent over lipase activity (15,40). We only used single buffer systems to observe the pH profile. For this reason, pH values under pH 5.5 cannot be considered reliable because of the pH lowering capacity of this buffer. Therefore, we chose a higher concentration of buffer to prevent drastic changes that might occur over activity, and for preventing activity loss originating from the ionic influence (that is, ionic strength) (67-70). The alkaline nature of *C. macrostomus* intestinal TG lipase, as indicated by a pH optimum between 6.5 and 9.0 (Table 2), is similar to other cytosolic acyl hydrolyses (71,72), including trout adipose tissue (45). Lipase activity associated with membrane elements and lysosomes occurs at pH 5.0 (acid lipase) (14,16). A major component of hepatic and intestinal lipase activity in mammals is alkaline (64). Intestinal acid lipase has also been detected in mammals (19), but knowledge about acid lipase similar to mammalian lipase, whether present or not in fish, is still controversial.

The apparent molecular mass of *Cyorinon macrostomus* intestinal lipase is 51 kDa by non-denaturing PAGE. The estimated molecular mass of trout hepatic TG lipase is similar to that of trout adipose TG lipase (48 kDa) (45) and that of chicken adipose TG lipase (42 kDa) (68). In rat adipose tissues, however, an 85-kDa protein has been demonstrated to possess TG lipase activity (69-71). In this manner, there is a similarity between *C. macrostomus* intestinal lipase and the other sort of lipase.

In conclusion, the intestinal TG lipase of *C. macrostomus* is probably similar to other TG lipases in some way such as molecular mass, hydrophobic character, and pH value, and this work describes the first partially purification of non-mammalian origin intestinal TG lipase using a different approach. The most remarkable result is that far more applications, including ion exchange, affinity chromatography, or gel filtration, can be carried out without having to remove the PEG. In our purification assay to some degree to remove the PEG from the protein solution is not necessary before chromatography, as supported by another investigation (62). Therefore the percentage of PEG-8000 used in the present study is not too detrimental for this application.

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