ELSEVIER

Journal of Chromatography B, 682 (1996) 233–242

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Inhibition of hepatic lipase by *m*-aminophenylboronate Application of phenylboronate affinity chromatography for purification of human postheparin plasma lipases

Mikko Uusi-Oukari, Christian Ehnholm, Matti Jauhiainen*

Department of Biochemistry, National Public Health Institute, Mannerheimintie 166, FIN-00300 Helsinki, Finland

Received 1 December 1995; revised 6 February 1996; accepted 6 February 1996

Abstract

Phenylboronates are competitive inhibitors of serine hydrolases including lipases. We studied the effect of *m*-aminophenylboronate on triglyceride-hydrolyzing activity of hepatic lipase (EC 3.1.1.3). *m*-Aminophenylboronate inhibited hepatic lipase activity with a K_i value of 55 μM. Furthermore, *m*-aminophenylboronate protected hepatic lipase activity from inhibition by di-isopropyl fluorophosphate, an irreversible active site inhibitor of serine hydrolases. Inhibition of hepatic lipase activity by *m*-aminophenylboronate was pH-dependent. The inhibition was maximal at pH 7.5, while at pH 10 it was almost non-existent. These data were used to develop a purification procedure for postheparin plasma hepatic lipase and lipoprotein lipase. The method is a combination of *m*-aminophenylboronate and heparin-Sepharose affinity chromatographies. Hepatic lipase was purified to homogeneity as analyzed on sodium dodecyl sulfate polyacrylamide gel electrophoresis. The specific activity of purified hepatic lipase was 5.46 mmol free fatty acids h^{-1} mg^{-1} protein with a total purification factor of 14 400 and a final recovery of approximately 20%. The recovery of hepatic lipase activity in *m*-aminophenylboronate affinity chromatography step was 95%. The purified lipoprotein lipase was a homogeneous protein with a specific activity of 8.27 mmol free fatty acids h^{-1} mg^{-1}. The purification factor was 23 400 and the final recovery approximately 20%. The recovery of lipoprotein lipase activity in the *m*-aminophenylboronate affinity chromatography step was 87%. The phenylboronate affinity chromatography step can be used for purification of serine hydrolases which interact with boronates.

Keywords: Enzymes; Lipases; *m*-Aminophenylboronate

1. Introduction

Hepatic lipase (HL; EC 3.1.1.3) is a lipolytic enzyme synthesized by hepatocytes [1–3] and localized mainly in hepatic sinusoids [4,5]. The enzyme has both triglyceride hydrolase and phospholipase A_1 activities [6]. It plays an important role in lipoprotein metabolism, primarily by hydrolyzing both intermediate-density lipoprotein (IDL) triglycerides to produce low-density lipoprotein (LDL) [7–9] and high-density lipoprotein$_2$ (HDL_2) triglycerides and phospholipids to produce HDL_3 [10–12], although the exact function of HL is still a matter of controversy [13,14].

Human HL is a glycoprotein containing 476 amino acids with a molecular mass of 65 000 [6,15]. It belongs to a family of lipases together with lipoprotein lipase (LPL) and pancreatic lipase (PL) [16–18].

*Corresponding author.

0378-4347/96/$15.00
PII S0378-4347(96)00082-5

The sequence homology between these three proteins in different animal species (29%) suggests both a similar tertiary structure of the proteins and a common evolutionary origin of the genes coding for the proteins [19–22]. The lipases are members of the serine esterase group of enzymes [23,24]. Boronates as competitive inhibitors of serine hydrolases [25,26] inhibit PL and LPL at micromolar concentrations [23,27–29].

Lipoprotein lipase (LPL; EC 3.1.1.34) plays a central role in lipoprotein metabolism, hydrolyzing both dietary and endogenous triglycerides transported in chylomicrons and very low density lipoprotein particles [30,31]. LPL is synthesized mainly in adipose tissue, muscle and lactating mammary gland [32]. It is secreted from parenchymal cells and transported to capillaries where it is bound to glycosaminoglycan components of the capillary endothelium [30,33]. Hydrolysis of triglycerides by LPL requires the presence of apolipoprotein C-II [34,35]. Another low-molecular-mass apolipoprotein, apo C-III, inhibits LPL in vitro [36,37]. LPL in the similar way to HL can be released into the circulation by intravenous injection of heparin enabling the measurement of its activity in post-heparin plasma [38].

Highly purified HL and LPL are required for in vitro studies of its role in lipoprotein metabolism and for preparation of specific antibodies. The methods used for HL purification have been reviewed in details [39], thereafter hydrophobic interaction chromatography in combination with heparin-Sepharose affinity chromatography has been mainly utilized for HL purification [40–43]. In the present study we have characterized the interaction of the serine hydrolase inhibitor *m*-aminophenylboronate (*m*-APB) with HL. This information was used to develop an affinity chromatography purification method for human postheparin plasma (PHP) HL and LPL.

2. Experimental

2.1. Materials

Glycerol tri[1-^{14}C]oleate (triolein) (specific activity 57 mCi mmol^{-1}) was purchased from Amersham International (Amersham, UK). Unlabelled glycerol trioleate, sorbitol, *m*-aminophenylboronate (*m*-APB) and *m*-APB immobilized on 6% beaded agarose via a nine-atom spacer (60 μmol boronate per ml of gel) were from Sigma (St. Louis, MO, USA). Heparin-Sepharose was from Pharmacia LKB (Uppsala, Sweden). Human HL-specific monoclonal antibody (3-6a) was a kind gift from Dr. Andre Bensadoun [15]. Diisopropyl fluorophosphate (DFP) was from Fluka (Buchs, Switzerland). *m*-APB and DFP were dissolved in dimethyl sulfoxide. *m*-APB was diluted in 10 m*M* phosphate buffer, pH 7.5 before use. The final concentration of dimethyl sulfoxide was 1% or less in all incubations, and it did not affect HL triglyceride lipase activity.

2.2. Methods

2.2.1. Enzyme assays

Triglyceride-hydrolyzing activity of hepatic lipase was measured in the presence of 1 *M* NaCl and in the absence or presence of various concentrations of *m*-aminophenylboronate (*m*-APB) using triolein as substrate essentially as described by Ehnholm and Kuusi [39]. The substrate was emulsified with 1% gum arabic in 0.1 *M* phosphate or 0.2 *M* Tris–HCl buffer, pH 8.5 [39], or with 0.04% (v/v) Triton X-100 in 0.1 *M* phosphate buffer, pH 8.5 [27]. Total triglyceride-hydrolyzing activity was measured as described above but in the presence of 0.15 *M* NaCl and 10% normal human serum. LPL activity was determined by subtracting HL activity from total activity [43].

2.2.2. Chemical modification

HL partially purified by heparin-Sepharose affinity chromatography was preincubated in the absence or presence of various *m*-APB concentrations at 24°C for 30 min. DFP was then added to the incubation mixtures at a final concentration of 2 m*M* and the incubation was continued for 1 h. As boronate and the hydroxyl groups of Tris [2-amino-2-(hydroxymethyl)-1,3-propanediol] form a complex that prevents the inhibitory effect of *m*-APB on HL activity, the activity of these samples were measured in 0.2 *M* Tris–HCl buffer, pH 8.5.

2.2.3. Purification of human postheparin plasma hepatic lipase

EDTA and aprotinin were added to 300 ml of human postheparin plasma to a final concentration of 1 m*M* and 50 U ml^{-1}, respectively. The plasma was applied onto a heparin-Sepharose column (40 ml) equilibrated with 10 m*M* phosphate buffer, pH 7.5 containing 0.15 *M* NaCl and 20% glycerol at a flow-rate of 1 $ml\,min^{-1}$. The column was then washed with 400 ml of the same buffer containing 0.4 *M* NaCl. Elution was performed with a linear NaCl gradient (0.4–2.0 *M*, 300 ml total volume). The fraction volume was 10 ml.

The peak fractions containing salt-resistant, but not salt-sensitive (LPL) triglyceride-hydrolyzing activity were combined, made 0.2% in Triton N-101 and applied onto an *m*-APB-agarose column (10.5× 1.6 cm I.D.) equilibrated with 10 m*M* phosphate, pH 7.5, containing 1 *M* NaCl, 10% glycerol, 1 m*M* EDTA and 0.2% Triton N-101. Flow-rate was 1 $ml\,min^{-1}$. The column was washed with 200 ml of equilibrating buffer containing 10 m*M* sorbitol and eluted with equilibrating buffer at various conditions: pH 6.0, pH 7.5 plus 0.3 *M* sorbitol, pH 9.0, pH 9.0 plus 0.3 *M* sorbitol, pH 10.0, and pH 10.0 plus 0.3 *M* sorbitol. The column was regenerated by 200 ml of equilibrating buffer, pH 7.5, without sorbitol.

The active fractions were combined, diluted 1:4 in 10 m*M* phosphate buffer, pH 7.5, containing 20% glycerol, 0.2% Triton N-101, and applied onto a second heparin-Sepharose column (affinity matrix volume: 2 ml) equilibrated as described above for the first heparin-Sepharose column at a flow-rate of 0.5 $ml\,min^{-1}$. HL was eluted with a linear NaCl gradient (0.4–1.2 *M*, 140 ml total volume) and 2-ml fractions were collected.

2.2.4. Purification of lipoprotein lipase

The fractions from first heparin-Sepharose column containing LPL activity were combined, made 0.2% by Triton N-101, diluted 1:5 in 10 m*M* phosphate, pH 7.5, containing 20% glycerol, and applied onto a second heparin-Sepharose column (2 ml) at a flow-rate of 0.5 $ml\,min^{-1}$. The column was washed with 50 ml of heparin-Sepharose column equilibrating buffer containing 0.8 *M* NaCl to wash the trace amounts of HL present in this pool. LPL was then eluted stepwise with 20 ml of heparin-Sepharose equilibrating buffer containing 2 *M* NaCl. This eluate was applied onto *m*-APB column, washed, and LPL activity subsequently eluted as described above for HL.

2.2.5. Other methods

SDS polyacrylamide gel electrophoresis was performed as described by Laemmli [44]. Western blotting was performed as described by Towbin et al. [45]. Protein concentration was determined according to a modification [46] of the Lowry method [47] using human serum albumin as a standard. The K_i values for inhibition of HL activity by *m*-APB were determined by linear, least-squares regression analysis using the computer program 'Kinetics' [48]. The K_m values of HL for triolein substrate used for calculation of K_i values were determined by non-linear, least-squares regression analysis of the same program using Marquardt–Levenberg algorithm. The K_i values were also determined by the method of Cleland [49].

3. Results

3.1. Inhibition of HL triglyceride-hydrolyzing activity by m-APB

3.1.1. Concentration-dependent inhibition

The triglyceride-hydrolyzing activity of HL in pooled fractions from heparin-Sepharose column was inhibited by *m*-APB in a dose-dependent manner (Fig. 1). The inhibition followed essentially first-order reaction kinetics and was almost identical whether gum arabic (GA) or Triton X-100 (TX-100) emulsified substrates were used. Using the equation for competitive inhibition $K_i=(K_m \cdot IC_{50}/(K_m+[S])$, linear regression analysis of the data resulted in an apparent inhibition constants (K_i) of 54.5±6.0 μM and 55.3±7.0 μM (mean±S.E.M., $n=3$) when assayed with GA and TX-100 emulsified substrates, respectively.

3.1.2. Effect of pH on the inhibition

Lipolytic activity was maximal at pH 9.0 with both substrate emulsions. No activity was detected

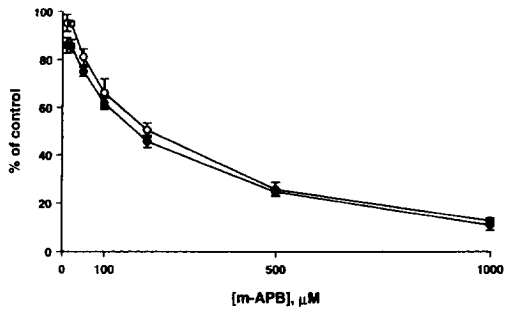

Fig. 1. Inhibition of hepatic lipase activity by *m*-aminophenylboronate. Pooled fractions of HL activity from a heparin-Sepharose column were incubated with gum arabic (closed circles) or Triton X-100 (open circles) emulsified triolein in the absence or presence of various concentrations of *m*-APB. Each point represents mean±S.E.M. of three determinations assayed in triplicate.

with TX-100 emulsified substrate at pH 6.0. The effect of pH on the inhibition of HL triglyceride-hydrolyzing activity by *m*-APB was measured in the presence of 200 μM *m*-APB, which at pH 7.5 resulted in approximately 50% inhibition (Fig. 1). As shown in Fig. 2, maximal inhibition was seen at pH 7.5 with both GA and TX-100 emulsified substrates;

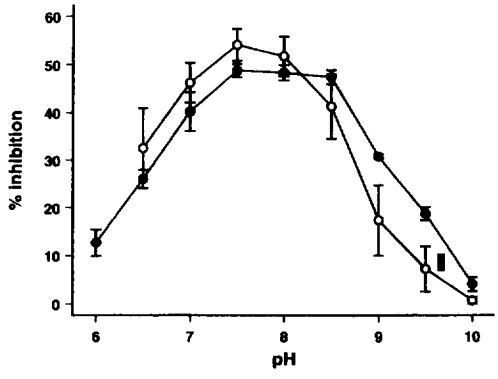

Fig. 2. Effect of pH on inhibition of hepatic lipase activity by *m*-aminophenylboronate. Pooled fractions of HL activity from a heparin-Sepharose column were incubated with gum arabic (closed circles) or Triton X-100 (open circles) emulsified triolein in the absence or presence of 200 μ*M* *m*-APB. Each point represents mean±S.E.M. of three determinations assayed in triplicate. The values are expressed as % inhibition as compared to samples incubated at the same pH without *m*-APB.

the inhibition was 40–55% in the pH range 7.0–8.5, and decreased sharply when the pH was shifted below or above this pH range with both substrates. At pH 10.0 the inhibition was only 4.0±1.4% and 0.67±0.67% (mean±S.E.M., $n=3$) using either GA or TX-100 emulsified substrates, respectively.

3.1.3. Protection of di-isopropyl fluorophosphate inhibition of HL triglyceride lipase activity by m-APB

HL triglyceride lipase activity in pooled fractions from first heparin-Sepharose column was completely inhibited by the presence of 2 m*M* DFP (Fig. 3). By preincubation of HL in the presence of 10 μ*M* *m*-APB the enzyme activity was protected from DFP

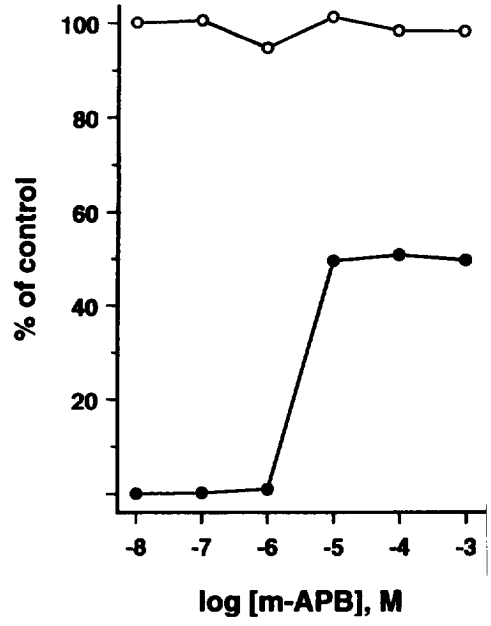

Fig. 3. Protection of hepatic lipase activity from di-isopropyl fluorophosphate inhibition by *m*-aminophenylboronate. Pooled fractions of HL activity from heparin-Sepharose column were incubated with (closed circles) or without (open circles) 2 m*M* DFP in the absence or presence of various *m*-APB concentrations as described in Section 2. After incubation, triglyceride-hydrolyzing activity was measured in 0.2 *M* Tris buffer, which prevents the inhibitory effect of *m*-APB on HL activity. The values represent the mean of two determinations assayed in triplicate.

Table 1
Purification of human postheparin plasma hepatic lipase

Purification step	Total protein (mg)	Total activity (mmol h^{-1})	Specific activity (mmol h)	Fold	Recovery (%)
Postheparin plasma	25 973	9.84	0.0004	1	100
I Heparin-Sepharose	48.9	4.87	0.10	263	49.5
m-Amino-phenylboronate	9.29	4.61	0.49	1310	46.9
II Heparin-Sepharose	0.352	1.92	5.46	14 400	19.5

Human postheparin plasma hepatic lipase was purified by three chromatography steps as described in Section 2. Triglyceride-hydrolyzing activity was measured using gum arabic emulsified triolein as substrate.

inhibition up to 50%. This protection was not increased further by increasing the concentration of *m*-APB up to 1 m*M*.

3.2. Purification of human postheparin plasma hepatic lipase

3.2.1. Heparin-Sepharose affinity chromatography

The salt-resistant (HL) and -sensitive (LPL) lipase activities of human postheparin plasma were separated by heparin-Sepharose affinity chromatography. HL activity was eluted in a range of 0.6–0.9 *M* NaCl; 49% of the activity that was applied to the column was recovered in combined peak fractions of HL activity (Table 1). The purification factor of this step was 263.

3.2.2. m-APB affinity chromatography

HL triglyceride-hydrolyzing activity partially purified by heparin-Sepharose affinity chromatography and subsequently applied onto a *m*-APB column was quantitatively bound to this affinity matrix. No activity was detected in flow through or in fractions eluted with 10 m*M* sorbitol in equilibrating buffer, pH 7.5 (Fig. 4). No activity was eluted by equilibrating buffer at pH 6.0 or pH 9.0, whereas at pH 10.0 HL activity was quantitatively eluted from the column. In the presence of 0.3 *M* sorbitol HL activity was quantitatively eluted at each pH-value tested. When eluted at pH 10 in the presence of 0.3 *M* sorbitol the elution gave the sharpest HL activity peak and these conditions were used for HL purification (Fig. 4). The recovery of the activity applied to the column was 95% and a purification factor of 5 was achieved as compared to the previous step.

3.2.3. Second heparin-Sepharose column

Active fractions eluted from the *m*-APB column were combined and concentrated using a second heparin-Sepharose column. At this step sorbitol was also removed from the sample. HL triglyceride-hydrolyzing activity was further purified by elution of the bound proteins from the column with a linear NaCl gradient (Fig. 5). The peak fractions containing HL activity were combined and subsequently used as a purified enzyme preparation. The total recovery after this step was 19.5% with a final purification factor of 14 400 (Table 1).

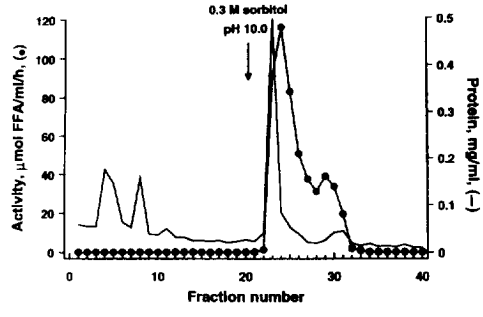

Fig. 4. Elution of hepatic lipase from *m*-aminophenylboronate column. The combined fractions of HL activity after heparin-Sepharose affinity chromatography were applied onto an *m*-APB column. After washing, bound proteins were eluted in 10 m*M* equilibrating buffer, pH 10.0, containing 0.3 *M* sorbitol.

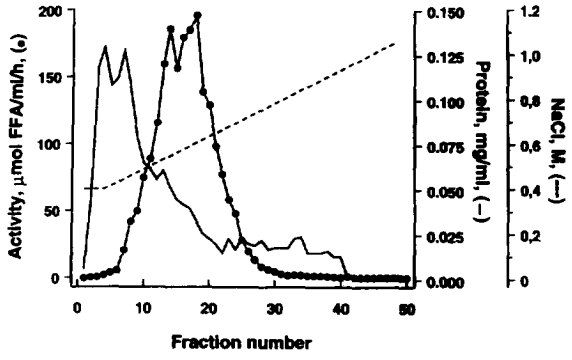

Fig. 5. Elution of hepatic lipase from second heparin-Sepharose column. The combined fractions of HL activity from *m*-APB column were applied onto heparin-Sepharose column. Bound proteins were eluted with a linear NaCl gradient (0.4–1.2 *M*).

3.2.4. SDS-polyacrylamide gel electrophoresis

Electrophoretic analysis of the proteins present in different purification steps revealed that most contaminating proteins, including antithrombin III, the major contaminant of heparin-Sepharose purified HL, did not bind to *m*-APB column (Fig. 6). Five distinct protein bands were visible in the *m*-APB column eluate. After the second heparin-Sepharose column the pooled HL activity fractions contained a major protein band with a molecular mass of 64 000 which was identified as HL by HL-specific monoclonal antibody, 3-6a, against human HL (Fig. 6).

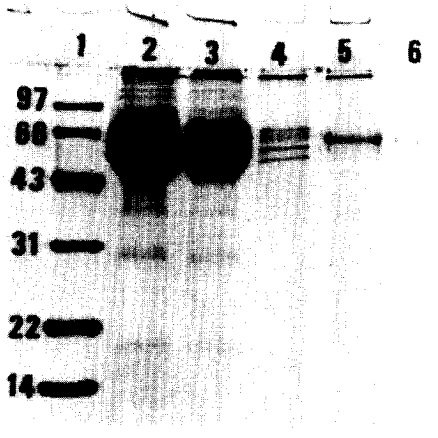

Fig. 6. SDS-polyacrylamide gel electrophoresis of hepatic lipase at various stages of purification. Protein samples were run on a 12.5% polyacrylamide gel and stained with Coomassie Blue. Lane 1, molecular mass markers, molecular mass in kDa; lane 2, I heparin-Sepharose eluate (60 µg); lane 3, *m*-APB flow-through (48 µg); lane 4, *m*-APB eluate (9 µg); lane 5, II heparin-Sepharose eluate (3 µg); lane 6, purified HL from lane 5 immunodetected by HL-specific monoclonal antibody.

3.3. Type of inhibition of HL triglyceride-hydrolyzing activity by m-APB

The interaction of *m*-APB with isolated HL was further characterized by Lineweaver–Burk analysis (Fig. 7). The analysis was performed using six substrate concentrations (0.25–6.0 m*M*) in the absence or presence of various *m*-APB concentrations. The reciprocal lines at inhibitor concentrations between 12.5–50 µ*M* intersected near 1/v axis suggesting a mixed but predominantly competitive type of inhibition (Fig. 7). At higher inhibitor concentrations (>100 µ*M*) there was a tendency towards a more mixed-type inhibition (data not shown). Cleland analysis of the inhibition gave an inhibition constant of 55 µ*M* (inset in Fig. 7).

3.4. Purification of human postheparin plasma lipoprotein lipase

LPL was purified from human postheparin plasma by two successive heparin-Sepharose columns and a *m*-APB column (Table 2). Of the different elution conditions tested, LPL eluted only at pH 10.0 in the presence of 0.3 *M* sorbitol in the equilibrating buffer. The purified enzyme had a specific activity of 8.27 mmol FFA h^{-1} mg^{-1} protein. The *m*-APB column step resulted in 7.5-fold purification with 86.5% recovery of LPL triglyceride-hydrolyzing activity as compared to the previous step. Total recovery of LPL was 19.3% with a final purification factor of 23 400. No salt-resistant HL activity or immunodetectable HL protein was present in purified LPL preparation.

4. Discussion

The hydrolysis of water-soluble substrates by pancreatic lipase (PL) proceeds via the classical acyl-enzyme mechanism of serine hydrolases involving the Ser-His-Asp proton relay system (see Ref. [50] for review). The catalytic triad of the PL active site has been localized with respect to the tertiary structure of the enzyme [21], and the individual

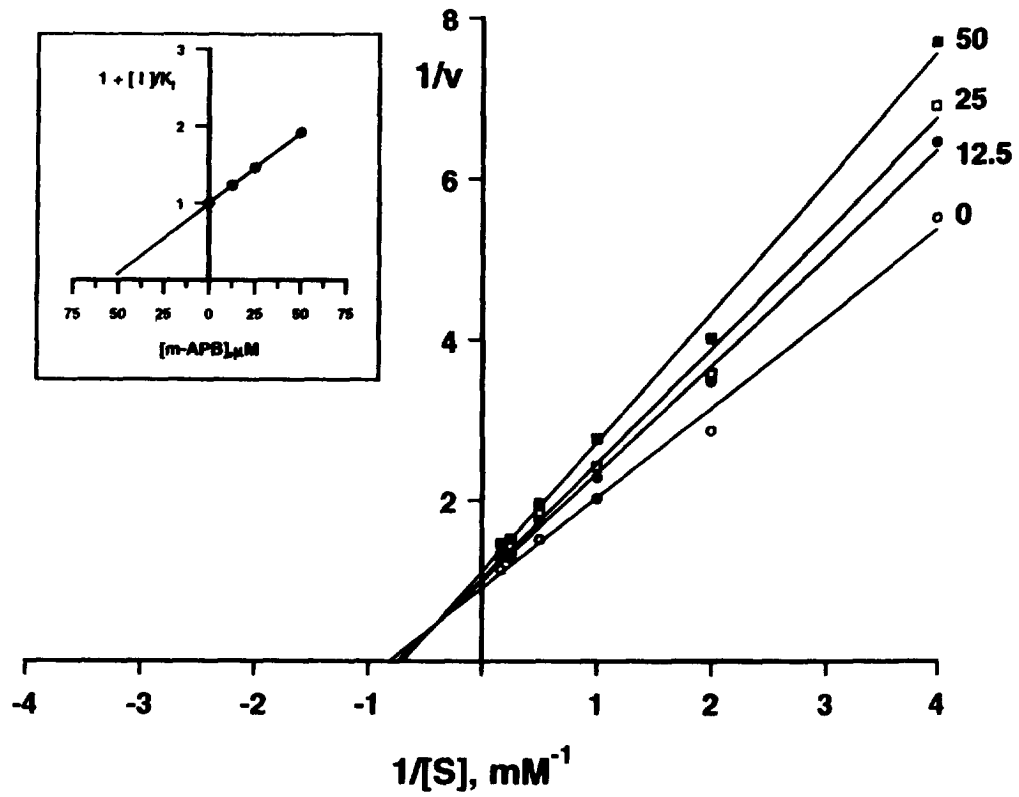

Fig. 7. Lineweaver–Burk plot of triglyceride-hydrolyzing activity of hepatic lipase in the absence or presence of indicated concentrations (μM) of *m*-aminophenylboronate. The activity was measured in the presence of increasing concentrations of triolein emulsified in gum arabic. The inset shows replots of the data for K_i determination according to the method of Cleland [49]. The values represent mean of two determinations assayed in triplicate.

amino acids of the triad have been confirmed by site-directed mutagenesis [51]. The mechanism of catalysis by LPL is less well established, but kinetic and inhibition studies of LPL activity suggest the participation of serine and histidine residues in catalysis and a catalytic mechanism that is general to serine-histidine hydrolases [50]. The catalytic triad of LPL has been confirmed by site-directed mutagenesis [52,53].

In contrast to PL and LPL [50], little is known about the mechanism of catalysis of HL. However, HL activity is inhibited by DFP and phenylmethyl sulfonylfluoride, modifiers of the catalytic triad serine [15,40,54]. Furthermore, the participation of

Table 2
Purification of human postheparin plasma lipoprotein lipase

Purification step	Total protein (mg)	Total activity (mmol h^{-1})	Specific activity (mmol h^{-1} mg^{-1})	Fold	Recovery (%)
Postheparin plasma	20 178	7.13	0.0004	1	100
I Heparin-Sepharose	9.64	2.60	0.27	765	36.5
II Heparin-Sepharose	1.44	1.59	1.10	3120	22.3
m-Amino-phenylboronate	0.166	1.37	8.27	23 400	19.3

Human postheparin plasma lipoprotein lipase was purified by three chromatography steps as described in Section 2. Triglyceride-hydrolyzing activity was measured using gum arabic emulsified triolein as substrate.

the proposed active site serine (Ser^{147}) in HL catalysis was strongly suggested by site-directed mutagenesis [55].

The mixed but predominantly competitive inhibition of HL activity by *m*-APB indicates that *m*-APB competes with triolein for the active site supporting the involvement of serine and histidine residues in the catalytic mechanism of HL. The interaction of HL and *m*-APB obviously results in the formation of a tetrahedral boronate adduct with the active site serine, as has been shown for the protease group of serine hydrolases [56,57]. An important feature of HL as well as of LPL is that the supposedly active site serine is completely buried by a loop or 'lid' which must be moved to give access to a substrate [58]. That *m*-APB can enter the active site is supported by the observations that a butylboronate adduct of the serine is formed in pancreatic lipase crystals [21]. The K_i value of *m*-APB for HL was in the low micromolar range as found previously for PL and LPL [23,27–29], suggesting similarity of the binding sites of these lipases as compared to low millimolar K_i values of serine proteases and lecithin-cholesterol acyltransferase (LCAT) for phenylboronates [25,59]. The reason for a mixed type of inhibition kinetics at higher phenylboronate concentrations is not known, but this phenomenon has been observed previously also for PL, LPL and LCAT [23,27,59]. As the lipases and LCAT are glycoproteins, the phenylboronates might bind to their carbohydrate structures, thereby non-competitively inhibiting enzyme activity. The dissociation constant of *m*-APB binding to mannitol is about 5 m*M* [23]. Therefore, at high concentrations phenylboronates are at least suggested to bind to carbohydrate structures of these proteins to some extent, although the effect of this binding is obscure. Vainio [60] showed that the presence of 10 m*M* mannitol had virtually no effect on the binding of low micromolar concentrations of dansyl-derivatized phenylboronate to LPL, thus confirming the active-site specificity of phenyl boronates.

DFP inhibited HL activity as found previously [15,40]. If we assume that HL is a homodimer with one catalytic site in both subunits as suggested for LPL [50], the 50% maximal protection of DFP inhibition by *m*-APB suggests that the binding of one molecule of *m*-APB to one active site of HL prevents the binding to the other site. Thus, the other site remains accessible for DFP inhibition. This interpretation, however, is in contradiction with the inhibitory effect of *m*-APB on HL activity, as the maximal inhibition of HL activity by *m*-APB was about 90%. *m*-APB might inhibit both active sites of HL dimer by binding to one site, but in this case the potency of *m*-APB to either inhibit HL activity (IC_{50}=200 μM) or protect binding sites against inactivation by DFP (IC_{50}=4 μM) is exerted in different concentration ranges. This controversy remains to be studied.

The reduced inhibitory potency of *m*-APB on HL activity at high pH was in accordance with its pK_a value of 9.2 [61]. The pH profile of the inhibition at pH range of 7–10 was similar to that found for PL using butane boronate as inhibitor [23]. The decrease at pH below 7 might be caused by ionization of the *m*-amino group in *m*-APB. Such an ionizable group is not present in butane boronate, which may explain its retained inhibitory action at pH below 7. However, no HL was eluted from *m*-APB column at pH 6 suggesting that the coupling of *m*-APB to matrix via amide linkage formed by *m*-amino group prevents its ionization. Therefore, HL remains bound to the column at pH 6.

m-APB affinity chromatography of HL was almost quantitative with respect to both retention of HL activity and recovery of the activity. The same was true for LPL. Similar recoveries using immobilized aminophenylboronate for purification have previously been achieved in the purification of serine proteases or PL [62,63], but these reports described only the phenylboronate purification step and not a whole purification protocol resulting in a pure, homogenous protein. The purification factors of 5 and 7.5 for HL and LPL, respectively, were not high, and the major purification steps were due to the two heparin-Sepharose columns. However, the main contaminant of PHP lipases using heparin-Sepharose affinity chromatography, antithrombin III, did not bind to *m*-APB column, and 10 m*M* sorbitol eluted most contaminant proteins from the column. In addition to HL, the four main proteins present in the *m*-APB column eluate might therefore represent different serine hydrolases, as their affinity to immobilized *m*-APB seems to be in the same range as that of HL. A specific advantage of phenylboronate affinity chro-

matography is that probably only the active form of the enzyme is bound, as suggested previously for PL [63].

The specific activities and recoveries of HL and LPL purified from PHP reported in this study are among the highest published thus far. Only four reports with significantly higher specific activity of HL (1.3–5.5 fold) have been published [15,42,43,64]. This difference might be explained by two reasons: (1) the volume of PHP as starting material in those studies is 9.3–70 fold greater; (2) triglyceride-hydrolyzing activity in most of those studies has been determined at 37°C where HL is more active than at the 28°C that was used in this study.

In conclusion, we have developed a purification protocol for PHP lipases using phenylboronate affinity chromatography in combination with heparin-Sepharose chromatography, which provides pure enzymes with high recovery and specific activity. As other lipases, including human milk bile-salt-stimulated lipase and various bacterial lipases are inhibited by phenylboronates [65,66], our phenylboronate affinity chromatography step may be a general tool used for purification of lipases and other serine hydrolases interacting with boronates.

Acknowledgments

The authors wish to thank Mrs Ritva Nurmi for expert technical assistance and Dr. Andre Bensadoun for providing the HL-specific monoclonal antibody. This study was supported by a grant from the Finnish Foundation for Cardiovascular Research.

References

[1] G.S. Sundaram, K.M.M. Shakir, G. Barnes and S. Margolis, J. Biol. Chem., 253 (1978) 7703–7710.
[2] H. Jansen, C. Kalkman, A.J. Zonneveld and W.C. Hülsmann, FEBS Lett., 98 (1979) 299–302.
[3] E.A. Laposata, H.M. Laboda, J.M. Glick and J.F. Strauss, III, J. Biol. Chem., 262 (1987) 5333–5338.
[4] T. Kuusi, E.A. Nikkilä, I. Virtanen and P.K.J. Kinnunen, Biochem. J., 181 (1979) 245–246.
[5] P.K.J. Kinnunen and I. Virtanen, in A.M. Gotto, L.C. Smith and B. Allen (Editors), Atherosclerosis, Vol. 5, Springer-Verlag, Berlin, 1980, pp. 383–386.
[6] C. Ehnholm, W. Shaw, H. Greten and W.V. Brown, J. Biol. Chem., 250 (1975) 6756–6761.
[7] T. Murase and H. Itakura, Atherosclerosis, 39 (1981) 293–300.
[8] I.J. Goldberg, N.A. Le, J.R. Paterniti and H.N. Ginsberg, J. Clin. Invest., 70 (1982) 1184–1192.
[9] B. Landin, A. Nilsson, J.S. Twu and M.C. Schotz, J. Lipid Res., 25 (1984) 559–563.
[10] T. Kuusi, P.K.J. Kinnunen and E.A. Nikkilä, FEBS Lett., 104 (1979) 384–388.
[11] H. Jansen, A. van Tol and W.C. Hülsmann, Biochem. Biophys. Res. Commun., 92 (1980) 53–59.
[12] K. Shirai, R.L. Barnhart and R.L. Jackson, Biochem. Biophys. Res. Commun., 100 (1981) 591–599.
[13] R.L. Jackson, in P.D. Boyer (Editor), Enzymes, Vol. 16, Academic Press, New York, 1983, pp. 141–181.
[14] P.K.J. Kinnunen, in B. Borgström and H.L. Brockman (Editors), Lipases, Elsevier, Amsterdam, 1984, pp. 307–328.
[15] C.-F. Cheng, A. Bensadoun, T. Bersot, J.S.T. Hsu and K.H. Melford, J. Biol. Chem., 260 (1985) 10720–10727.
[16] S. Datta, C.-C. Luo, W.-H. Li, P. van Tuinen, D.H. Ledbetter, M.A. Brown, S.-H. Chen, S.-W. Liu and L. Chan, J. Biol. Chem., 263 (1988) 1107–1110.
[17] K.L. Wion, T.G. Kirchgessner, A.J. Lusis, M.C. Schotz and R.M. Lawn, Science, 235 (1987) 1638–1641.
[18] M.E. Lowe, J.L. Rosenblum and A.W. Strauss, III, J. Biol. Chem., 264 (1989) 20042–20048.
[19] T.G. Kirchgessner, K.L. Svenson, A.J. Lusis and M.C. Schotz, J. Biol. Chem., 262 (1987) 8463–8466.
[20] D. Ameis, G. Stahnke, J. Kobayashi, J. McLean, G. Lee, M. Büscher, M.C. Schotz and H. Will, J. Biol. Chem., 265 (1990) 6552–6555.
[21] F.K. Winkler, A. D'Arcy and W. Hunziker, Nature, 343 (1990) 771–774.
[22] B. Persson, H. Jörnvall, T. Olivecrona and G. Bengtsson-Olivecrona, FEBS Lett., 288 (1991) 33–36.
[23] C.W. Garner, J. Biol. Chem., 255 (1980) 5064–5068.
[24] P.K.J. Kinnunen, J.A. Virtanen and P. Vainio, in A.M. Gotto Jr. and R. Paoletti (Editors), Atherosclerosis Reviews, Vol. 11, Raven Press, New York, 1983, pp. 65–105.
[25] R. Wolfenden, Annu. Rev. Biophys. Bioeng., 5 (1976) 271–306.
[26] J. Kraut, Annu. Rev. Biochem., 46 (1977) 331–358.
[27] P. Vainio, J.A. Virtanen and P.K.J. Kinnunen, Biochim. Biophys. Acta, 711 (1982) 386–390.
[28] D.M. Quinn, Biochemistry, 24 (1985) 3144–3149.
[29] L.D. Sutton, J.S. Stout, L. Hosie, P.S. Spencer and D.M. Quinn, Biochem. Biophys. Res. Commun., 134 (1986) 386–392.
[30] R.H. Eckel, N. Engl. J. Med., 320 (1989) 1060–1068.
[31] M.-R. Taskinen and T. Kuusi, Bailliere's Clin. Endocrinol. Metabolism, 1 (1987) 638–666.
[32] J.E.A. Braun and D.L. Severson, Biochem. J., 287 (1992) 337–347.
[33] T. Olivecrona and G. Bengtsson-Olivecrona, Curr. Opin. Lipidol., 1 (1990) 116–122.
[34] C.-S. Wang, J. Hartsuck and W.J. McConathy, Biochim. Biophys. Acta, 1123 (1992) 1–17.

[35] S. Santamarina-Fojo, Curr. Opin. Lipidol., 3 (1992) 186–195.
[36] R.M. Krauss, P.N. Herbert, R.I. Levy and D.S. Fredrickson, Circulation Res., 38 (1973) 403–411.
[37] W.J. McConathy, J.C. Gesquiere, H. Bass, A. Tartar, J.-C. Fruchart and C.-S. Wang, J. Lipid Res., 33 (1992) 995–1003.
[38] J.K. Huttunen, C. Ehnholm, P.K.J. Kinnunen and E.A. Nikkilä, Clin. Chim. Acta, 63 (1975) 335–347.
[39] C. Ehnholm and T. Kuusi, Methods Enzymol., 129 (1986) 716–738.
[40] J.-S. Twu, A.S. Garfinkel and M.C. Schotz, Biochim. Biophys. Acta, 792 (1984) 330–337.
[41] H. Kresse, B.-M. Löffler and H. Kunze, Biochem. Int., 23 (1991) 885–893.
[42] Y. Ikeda, A. Takagi and A. Yamamoto, Biochim. Biophys. Acta, 1003 (1989) 254–269.
[43] R.L. Jackson and L.R. McLean, Methods Enzymol., 197 (1991) 339–345.
[44] U.K. Laemmli, Nature, 227 (1970) 680–685.
[45] H. Towbin, T. Staehelin and J. Gordon, Proc. Natl. Acad. Sci. USA, 76 (1979) 4350–4357.
[46] G.L. Peterson, Anal. Biochem., 83 (1977) 346–356.
[47] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265–275.
[48] S.P.J. Brooks, Biotechniques, 13 (1992) 906–911.
[49] W.W. Cleland, Biochim. Biophys. Acta, 67 (1963) 173–187.
[50] P.J. Dolphin, in M. Rosseneu (Editor), Structure and Function of Apolipoproteins, CRC Press, Boca Raton, FL, 1992, pp. 295–360.
[51] M.E. Lowe, J. Biol. Chem., 267 (1992) 17069–17073.
[52] F. Faustinella, L.C. Smith, C.F. Semenkovich and L. Chan, J. Biol. Chem., 266 (1991) 9481–9485.
[53] J. Emmerich, O.U. Beg, J. Peterson, L. Previato, J.D. Brunzell, H.B. Brewer and S. Santamarina-Fojo, J. Biol. Chem., 267 (1992) 4161–4165.
[54] M.C. Komaromy and M. Reed, J. Lipid Res., 32 (1991) 963–975.
[55] R.C. Davis, G. Stahnke, H. Wong, M.H. Doolittle, D. Ameis, D. Will and M.C. Schotz, J. Biol. Chem., 265 (1990) 6291–6295.
[56] K.A. Koehler and G.E. Lienhard, Biochemistry, 10 (1971) 2477–2483.
[57] D.A. Matthews, R.A. Alden, J.J. Birktoft, S.T. Freer and J. Kraut, J. Biol. Chem., 250 (1975) 7120–7126.
[58] J.-M. Lalouel, D.E. Wilson and P.-H. Iverius, Curr. Opin. Lipidol., 3 (1992) 86–95.
[59] M. Jauhiainen, N.D. Ridgway and P.J. Dolphin, Biochim. Biophys. Acta, 918 (1987) 175–188.
[60] P. Vainio, Biochim. Biophys. Acta, 746 (1983) 217–219.
[61] R.R. Maestas, J.R. Prieto, G.D. Kuehn and J.H. Hageman, J. Chromatogr., 189 (1980) 225–251.
[62] V. Akparov Kh. and V.M. Stepanov, J. Chromatogr., 155 (1978) 329–336.
[63] V. Akparov Kh., N.N. Nutsubidze and T.V. Romanova, Sov. J. Bioorg. Chem., 6 (1981) 317–320.
[64] A.-M. Österlund-Lindqvist and J. Boberg, FEBS Lett., 83 (1977) 231–236.
[65] N. Abouakil and D. Lombardo, Biochim. Biophys. Acta, 1004 (1989) 215–220.
[66] A. Köpf, S. Schalkhammer, G. Kirchner and F. Pittner, Biocatalysis, 9 (1994) 261–268.