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A protocol for the combined sub-fractionation and delipidation of lipid binding proteins using hydrophobic interaction chromatography

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ABSTRACT

Cellular lipids frequently co-purify with lipid binding proteins isolated from tissue extracts or heterologous host systems and as such hinder *in vitro* ligand binding approaches for which the *apo*-protein is a prerequisite. Here we present a technique for the complete removal of unesterified fatty acids, phospholipids, steroids and other lipophilic ligands bound to soluble proteins, without protein denaturation. Peroxisome proliferator activated receptor γ ligand binding domain and intracellular fatty acid binding proteins were expressed in an *Escherichia coli* host and completely delipidated by hydrophobic interaction chromatography using phenyl sepharose. The delipidation procedure operates at room temperature with complete removal of bound lipids in a single step, as ascertained by mass spectrometry analysis of organic solvent extracts from purified protein samples. The speed and capacity of this method makes it amenable to scale-up and high-throughput applications. The method can also easily be adapted for other lipid binding proteins that require delipidation under native conditions.

1. Introduction

Intracellular long-chain fatty acids (FA) are key components in the synthesis of cellular membranes as well as being utilized as signaling molecules and for energy delivery [1]. The preservation of a proper balance between absorption, secretion, and storage of FA, is therefore, integral for cellular physiology. Increasingly prominent diseases such as obesity, cardiovascular diseases, type II diabetes, and atherosclerosis, to a large extent, all evolve from disorders of lipid metabolism [2]. Due to their poor solubility in water, transport of FA *in vivo* is via intracellular lipid binding proteins (iLBPs) [1,3,4]. The expression of genes involved in FA metabolism and glucose homeostasis is controlled by nuclear receptors, in particular a class termed the peroxisome proliferator-activated receptors (PPARs) [5–9]. PPARs are ligand-activated transcription factors that are activated by FA and eicosanoids [5–9].

Three isotypes of human PPAR, termed α, γ and δ, have been identified each with a specific tissue distribution [5–9]. PPARα and γ are the most studied isotypes. PPARα modulates FA metabolism and glucose homeostasis in the liver and skeletal muscle, whereas PPARγ modulates adipogenesis and adipocyte FA metabolism [5–9]. The physiological role of PPARδ is the least understood of the three human PPAR isotypes. However, not unlike the other two isotypes, PPARδ binds FA and eicosanoids, signifying a regulatory role in lipid metabolism [9]. Dysfunction of these aspects of biology leads to the aforementioned human diseases. Accordingly, PPARs are important targets for anti-dyslipidemic drugs [7,8].

The intracellular trafficking mechanisms whereby lipid signaling molecules reach their nuclear receptor targets are not precisely known. Available evidence suggests the most likely candidates are a family of low molecular weight (12–15 kDa) iLBPs, collectively termed fatty acid binding proteins (FABPs) [1,3,4]. FABPs appear to act as intracellular shuttles for lipophilic ligands to the nucleus, where the ligand is released to PPARs, thereby effecting transcriptional regulation of metabolic enzymes and transporters that target the activating ligand [10–13].

In light of the central regulatory role of PPARs and FABPs in lipid homeostasis, it follows that the development of novel therapeutic ligands with improved pharmacological profiles to target these iLBPs, has become an important research priority in the pharmaceutical industry [2,7,8]. However, the study of iLBP ligand binding affinities and the molecular interactions governing ligand selectivity are complicated due to the co-purification of high affinity endogenous lipids. Current protein delipidation methods

Abbreviations: ANS, 1-anilino-8-naphthalene sulfonic acid; bisANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid; FABP, fatty acid binding protein; HAP, hydroxyalkylpropyl; HIC, hydrophobic interaction chromatography; iLBP, intracellular lipid binding protein.

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are far from optimal, often operating under denaturing conditions. Research into the development of improved delipidation methodology that can be implemented on an industrial scale is severely lacking. Our interest in the characterization of PPAR and FABP ligand interactions lead us to test two commonly employed procedures for the delipidation of iLBPs. The organic solvent liquid–liquid extraction [14,15] and lipidex 1000 [16–18] methods were compared to a novel hydrophobic interaction chromatography (HIC) procedure in order to develop new and improved delipidation methodology for aqueous lipid binding proteins. The presented HIC protocol combines delipidation with sub-fractionation of aqueous lipid binding proteins without denaturation of native protein structure.

2. Materials and methods

2.1. Materials

Oleic acid, [1-^{14}C], (54.6 mCi/mmol) was purchased from MP Biomedicals Australia (Seven Hills, N.S.W., Australia). 1-Anilino-8-naphthalene sulfonic acid (ANS), 8,8′-dianilino-5,5′-binaphthalene-1,1′-disulfonate (bisANS) and FA standards were obtained from Sigma–Aldrich (Sydney, NSW, Australia). *Cis*-parinaric acid was purchased from Invitrogen (Melbourne, VIC, Australia). *Escherichia coli* (*E. coli*) strain BL21 Codon Plus (DE3)-RIL was purchased from Stratagene (La Jolla, CA, USA). The following *E. coli* expression plasmids were generously donated by these researchers: human ileal bile acid binding protein (I-BABP) from Ira Ropson, Penn State University [19]; the ligand binding domain (LBD) aa193-475 of human PPARγ from Krister Bamberg, Department of Molecular Biology, Astra-Zeneca R&D Mölndol [20]; the *E. coli* expression plasmid for human L-FABP was developed internally and is available from the Plasmid Repository (http://dnaseq.med.harvard.edu/plasmid_repository.htm) under the Plasmid identification code: HsCD00073511. All other reagents were of the highest purity commercially available.

2.2. Expression and purification of recombinant proteins

The purification schemes and buffer conditions employed in this study are detailed in Supplementary Fig. 1 and Table 1, respectively. All proteins were expressed in *E. coli* BL21 cells as previously described [19–21]. In brief, human I-BABP was purified by HIC coupled to an anion exchange step as previously described [21]. Additionally, in order to isolate protein that had not been subjected to HIC, a separate I-BABP purification was performed by a method previously described by the Ira Ropson laboratory [19]. The human PPARγLBD, and human L-FABP were engineered with a N-terminal $[His]_6$ affinity tag and were separated from the bulk contaminants in the soluble cell fraction by Ni^{2+}-based immobilized metal ion affinity chromatography (IMAC) on a Ni^{2+} Sepharose 5 ml HisTrapHP chromatography column (GE Health Care, Sydney, N.S.W., Australia, Cat#17-5248-02). Proteins were resolved using a step gradient of 0–300 mM imidazole in buffer A at a flow rate of 5 ml/min (four column volumes (CV) wash-out unbound sample; 0–30% imidazole over five CV; hold 30% for two CV; 30–100% imidazole over five CV; hold 100% for three CV). Partially purified proteins were brought to 18% (PPARγLBD) or 65% saturation (I-BABP and L-FABP) with ammonium sulfate and stirred slowly for 1 h at 4 °C. Delipidation and further purification was achieved by HIC on a Phenyl HP 16/10 column (GE Health Care, Sydney, N.S.W., Australia, Cat#17-1085-01). Protein samples were applied to the column at a temperature of 20 °C and eluted with a linear gradient of 100–0% 1.0 M ammonium sulfate in buffer B at a flow rate of 3 ml/min over three CV. The final purity of the proteins was ascertained by SDS-PAGE (silver staining) and in all cases was >98%.

2.3. Delipidation by 1-butanol liquid–liquid extraction

Purified proteins were delipidated by extraction three times with 1/3 volume of 1-butanol [15]. The emulsion was centrifuged for phase separation and the 1-butanol layer was removed by vacuum centrifuge.

2.4. Delipidation by lipidex 1000 chromatography

Delipidation by the lipidex 1000 (a hydroxyalkylpropyl (HAP)) derivative of sephadex G25 substituted 10% with alkyl chains of C_{15}–C_{18} in length *syn.* (HAP)-dextran type VI *syn.* Sephadex LH 20-100; Sigma–Aldrich Cat#H-6258) method was performed as previously described [16–18]. Briefly, the sample was applied to a 15 ml column of lipidex 1000 pre-equilibrated with buffer C at 37 °C, and eluted at a flow rate of 15 ml/h. The column temperature and all solutions were maintained at 37 °C throughout the procedure. The non-derivatized resin, sephadex LH-20 (hydroxypropylated cross-linked dextran, prepared by hydroxypropylation of sephadex G-25; GE Health Care Cat#17-0090-01) was also tested for delipidation efficacy under the same conditions.

2.5. Quantitative assessment of the degree of delipidation

Purified protein samples that have been delipidated by HIC were incubated with 5 nmol of oleic acid, [1-^{14}C], (54.6 mCi/mmol) for 30 min at 20 °C. The ammonium sulfate content of the samples was then adjusted as described above and samples were subjected to HIC. Radioactivity was assayed by liquid scintillation counting of 200 μl samples from each column fraction.

2.6. Electrospray ionization mass spectrometry (ESI-MS) characterization of protein-bound lipids

All glassware was washed with 3 M nitric acid and sonicated, clean gloves were worn at all times to avoid contamination from exogenous lipid. For lipid extraction from purified protein samples, a phase separation was induced by adding a 1:1 volume of ice-cold HPLC grade ethyl acetate to purified protein solution. After vigorous vortexing, the emulsion was incubated at −20 °C for 15 min and centrifuged for 10 min at 13,000 × *g* at 4 °C. Protein became insoluble and formed a layer between the ethyl acetate top phase and the bottom phase consisting mainly of water and buffer salts. The top phase was removed with a capillary tip above the inter-phase to avoid contamination. Ethyl acetate extracts were analyzed by ESI-MS on a Micromass Platform II liquid chromatography quadra pole mass spectrometry system (Manchester, UK). Approximately 100 μl of ethyl acetate extract was directly injected into the mass spectrometer. Data were collected in negative ion mode at cone voltages of 20, 50 and 100 electron volts (eV). The injection port of the spectrometer was cleaned before and after each application and solvent only scans were performed to ascertain there was no sample-to-sample carry over contamination. The experimental masses of lipid compounds were referenced against FA standards, lipid mass spectra in the literature and the LIPIDMAPS structural database ([22–24]; http://www.lipidmaps.org).

2.7. Chemical synthesis of 8,8′-dianilino-5,5′-binaphthalene-1,1′-disulfonate (bisANS)

bisANS was synthesized according to a modified literature procedure [25] described in detail in the supplementary information.

2.8. Ligand binding fluorescence measurements

Fluorometric protein-ligand binding affinity measurements were performed under steady-state conditions on a Cary Eclipse fluorescence spectrophotometer in buffer D at 20 °C (Varian, Mulgrave, Victoria, Australia). Fluorometric titrations of ANS into L-FABP were performed as previously described [26]. BisANS was used as the binding cavity probe for human I-BABP [27]. The binding properties of PPARγLBD were measured by titration with the fluorescent FA *cis*-parinaric acid as previously described [28]. The functionality of PPARγLBD was also examined by measuring the binding of the synthetic agonist GW1929, with know high PPARγ sub-type specificity [29]. Competition experiments were performed where PPARγLBD (4 μM) was pre-incubated with *cis*-parinaric acid (300 nM) and titrated with GW1929. The decrease in *cis*-parinaric acid fluorescence upon addition of GW1929 was monitored and plotted as a function of the concentration of competing ligand. The data were fitted by non-linear regression to a one-site-competition model from which EC_{50} values were derived. The inhibition constant (K_i) for GW1929 binding was calculated according to the equation $K_i = EC_{50}/(1 + [\textit{cis}\text{-parinaric acid}]/K_{d\ \textit{cis}\text{-parinaric acid}})$. All other data modeling operations were performed as previously described [26–28] using GraphPad Prism V4.0 software (GraphPad software, San Diego, CA, USA).

2.9. Differential scanning calorimetry (DSC)

DSC measurements of the thermal stability of the *apo*-form of the three iLBPs were carried out on a Model 6100 N-DSC II differential scanning microcalorimeter (Calorimetry Sciences Corporation, Spanish Fork, UT, USA). For all DSC scans, proteins were dialyzed against sample buffer D and a base-line was determined using this dialysis buffer. Prior to being loaded into the microcalorimeter cell, samples were centrifuged at 13,000 × *g* and filtered to remove any particulate matter. A scan rate of 1 °C/min was used for base-line and sample scans. Protein concentrations were 91.0 μM (PPARγLBD), 320.0 μM (L-FABP) and 527.0 μM (I-BABP). The raw data were converted to listings of excess specific heat. After concentration normalization and base-line correction, analysis and fitting of the thermograms to a two-state transition model were performed using the 6100 N-DSC II software package (Calorimetry Sciences Corporation, Spanish Fork, UT, USA). The thermodynamic stability parameters: thermal unfolding midpoint temperature (t_m), the calorimetric enthalpy (ΔH_{cal}) and entropy (ΔS_{cal}) of unfolding were calculated from the transition curves.

2.10. Protein concentration determination

Protein concentration was determined by the Bradford dye binding method and by UV–vis spectrometry using the extinction coefficient at 280 nm of each protein calculated from the amino acid sequence: PPARγLBD aa196-468 (10,430 M^{-1} cm^{-1}), human I-BABP (11,460 M^{-1} cm^{-1}) and human L-FABP (1490 M^{-1} cm^{-1}).

3. Results and discussion

3.1. Protein purification and characterization of protein-bound endogenous lipids

E. coli cells are routinely employed for the expression of mammalian proteins, owing to the simple and inexpensive culturing conditions, in addition to the high yields of recombinant protein produced. In our laboratory we employ *E. coli* BL21 cells for the expression of human and rat FABPs and PPAR LBDs and have established purification protocols [21]. The Ni^{2+}-based IMAC purification of N-terminally $[His]_6$ tagged human L-FABP and PPARγLBD, and HIC/anion exchange purification of I-BABP are given as an examples (Supplementary Figs. 1 and 2). The $[His]_6$ tagged proteins could be resolved to near electrophoretic homogenity (silver-staining) using an IMAC step gradient. Contaminants could be eliminated in the unbound eluant and the 0–30% imidazole fractions, whereas the recombinant protein eluted at high purity in the 30–100% imidazole fraction. Human I-BABP was purified to homogeneity by two methods, (1) HIC followed by anion exchange chromatography [21]. (2) Anion exchange followed by Superdex G75 filtration [19]. I-BABP purified by the latter method was not subjected to delipidation during the chromatography process and was employed for comparative testing of the three delipidation methods described hereon in. Negative-ion ESI-MS analysis of ethyl acetate extracts of purified protein samples indicated a number of non-covalently bound lipids acquired from the bacterial expression system co-purify with each protein (Fig. 1). The spectra of extracts from human L-FABP show the presence of anions predicted for C12:1 (*m*/*z* 197), C16:0 (*m*/*z* 255), C16:1 (*m*/*z* 253), C18:0 (*m*/*z* 283) and C18:1 (*m*/*z* 281) FA, with C16:0 and C18:1 as the predominant species. The lipid profile observed for recombinant human L-FABP is consistent with that reported for recombinant rat L-FABP also derived from *E. coli* cells [30] and that of native L-FABP purified from rat liver homogenate [31].

The spectra of extracts from I-BABP purified by method 2 indicated the presence of a similar mixture of lipids to those observed bound to the L-FABP sample. The most abundant FA species were C12:1 (*m*/*z* 197), C16:0 (*m*/*z* 255), 18:0 (*m*/*z* 283) and C18:1 (*m*/*z* 281). A similar lipid profile was observed for recombinant mouse I-BABP produced in *E. coli* [32]. The spectra of I-BABP purified by method 1 did not show the presence of lipids as this procedure incorporates a HIC step (*cf* Fig. 1D, right panel). The PPARγLBD extracts contained C16:0 (*m*/*z* 255) and 18:0 (*m*/*z* 283) FA, with C18:0 as the predominant species, similar results have been reported for PPARδLBD expressed in *E. coli* [33].

In summary, mass spectrometry analyses of organic extracts from the recombinant iLBPs indicates these proteins are loaded with FA and other lipidic compounds originating from the bacterial expression system. This observation would suggest some ligands that have been tested against recombinant proteins prepared from an *E. coli* host may actually be more avid at binding than reported. Thus, binding data accumulated on the association of recombinant lipid binding proteins with ligands must be viewed with caution unless the protein has been delipidated prior to binding measurements.

3.2. Comparison of protein delipidation methods

HIC delipidation of the purified proteins was compared to two established methods commonly employed for the delipidation of iLBPs, 1-butanol liquid–liquid extraction [14,15] and lipidex 1000

Table 1
Recovery yields of soluble purified protein following delipidation treatment

	Protein recovery (mg)[a]		
Protein	HIC delipidation	1-Butanol delipidation (1:3; 1-butanol:protein volume)	Lipidex 1000 delipidation at 37 °C
L-FABP	2.8 ± 0.2	2.0 ± 0.4	2.8 ± 0.1
I-BABP	2.8 ± 0.1	2.1 ± 0.7	2.8 ± 0.2
PPARγLBD	2.9 ± 0.1	1.8 ± 0.5	2.0 ± 0.3

A total of 3 mg of each protein was applied to each delipidation treatment.

[a] Determined by UV–vis spectrometry using the extinction coefficient for each protein, mean values from three independent experiments are indicated ± standard deviation.

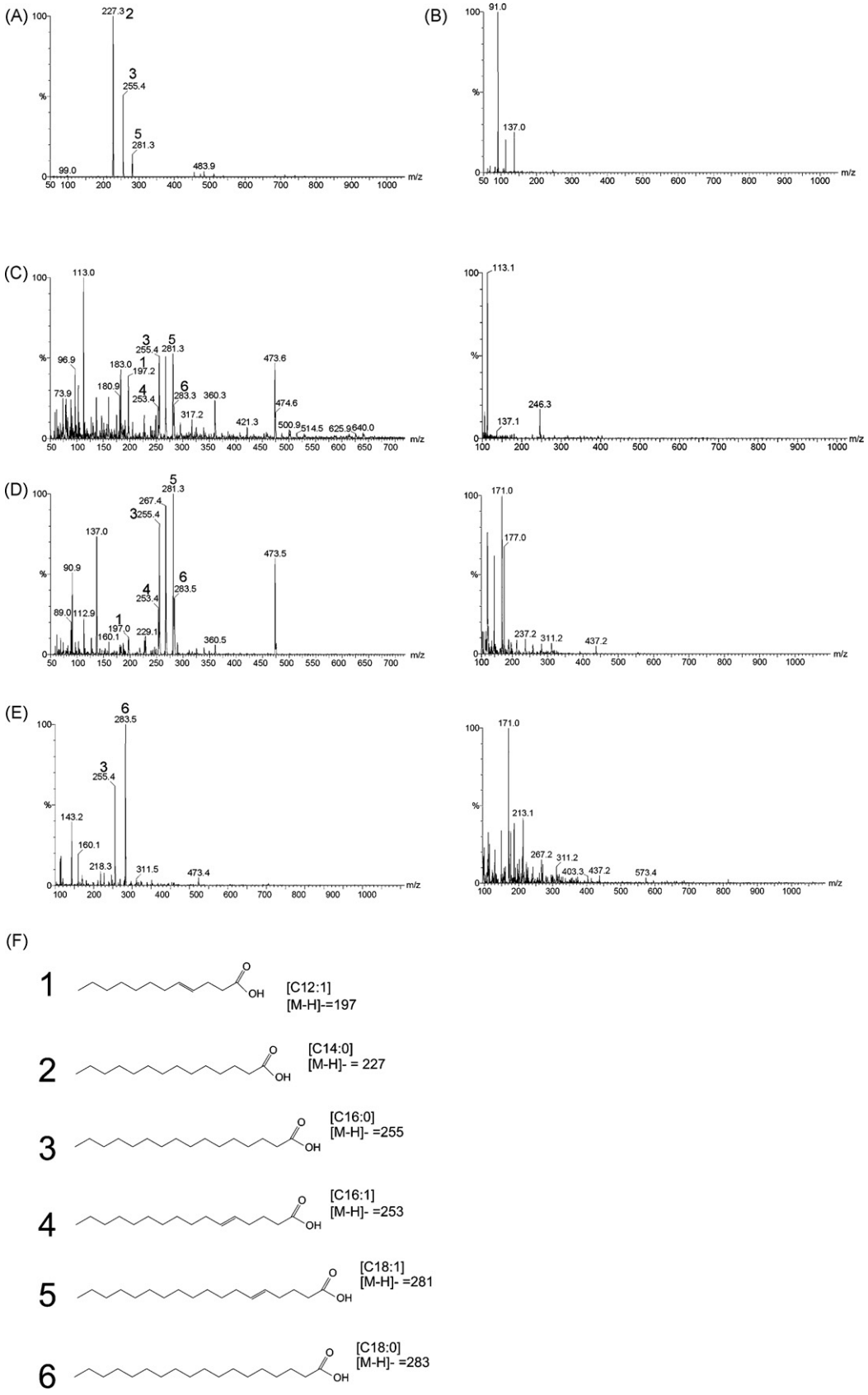

Fig. 1. ESI-MS analysis of endogenous lipids bound to recombinant iLBPs isolated from an *E. coli* host prior to delipidation. FAs were extracted into ethyl acetate and analysed by negative ion ESI-MS. (A) Mixture of control FAs. (B) Ethyl acetate solvent control. (C–E) ESI-MS spectra of ethyl acetate extracts from native (left panels) and following HIC delipidation (right panels) (C) human L-FABP (D) human I-BABP (E) human PPARγLBD. (F) Chemical structure of endogenous *E. coli* lipids. Compounds are assigned numerically, and are indicated accordingly on mass spectra. The mass of the negative ion, chain length and saturation states are indicated.

[16–18]. A direct comparison of each method in terms of the recovery of soluble purified protein applied is documented in Table 1. The data show that the HIC method provides an almost quantitative recovery of purified protein for all three proteins. In comparison, the lipidex 1000 method resulted in a significant loss of PPARγLBD, whereas I-BABP and L-FABP recovery levels were similar to HIC. The 1-butanol treatment resulted in a significant protein loss in each case. The recovery of additional protein with the HIC method presents significant advantages for structural NMR studies that require $^{2}H/^{15}N/^{13}C$ triple isotopically labeled protein samples, as this comes at a considerable expense.

In order to test the effectiveness of the HIC method as a combined sub-fractionation/delipidation process, the partially purified proteins were applied to a phenyl sepharose HIC column at 20 °C (Fig. 2). The respective 30–100% imidazole fractions from Ni^{2+}-based IMAC purification of L-FABP and PPARγLBD were pooled and applied to HIC (Supplementary Figs. 1 and 2). Whereas, with the I-BABP purification strategy, HIC was employed to resolve the I-BABP containing soluble fraction of the 65% ammonium sulfate cut of the *E. coli* cell lysate (Supplementary Figs. 1 and 2). L-FABP and I-BABP were completely absorbed onto the HIC support when applied in a 3.0 M (65% saturation) ammonium sulfate buffer, whereas 1.0 M (18% saturation) was used for PPARγLBD. HIC effectively resolved contaminant proteins and all iLBPs eluted in the ammonium sulfate-free fraction of the elution gradient (Fig. 2). Lipids were not detectable in ESI-MS spectra of ethyl acetate extracts from each protein following a single HIC treatment (Fig. 1C–E, right panels). A quantitative assessment of the degree of delipidation afforded by each method were performed by following the proportion of protein-bound radioactive tracer FA, [^{14}C]-Oleate (Fig. 3; Table 2). HIC achieved almost complete delipidation following a single treatment. The total amount of radioactivity recovered was not quantitative, so it appears a proportion of the tracer FA non-specifically absorbs onto the surface of plastic test-tubes. Fig. 3 shows the protein elution profile monitored by SDS-PAGE against the counts of tracer FA released with each HIC fraction. Most of the FA tracer remained strongly bound to the HIC resin following elution of the iLBP and only desorbed when the eluant was changed to methanol (data not shown).

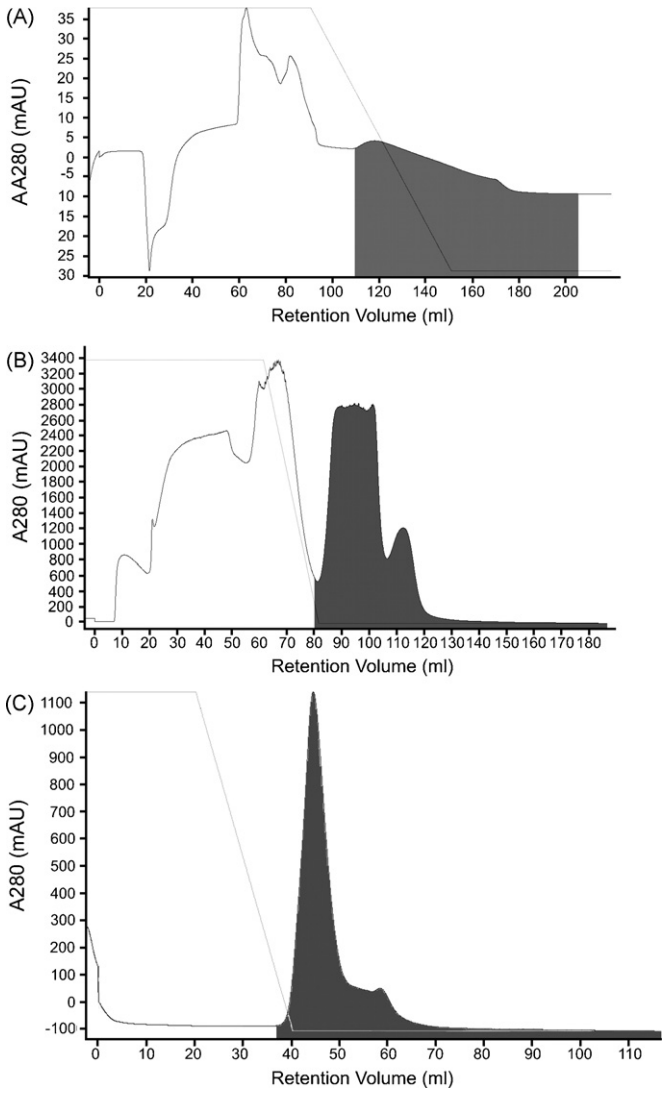

Fig. 2. HIC delipidation/sub-fractionation of recombinant iLBPs. Chromatograms of the phenyl sepharose elution profile of (A) human L-FABP (B) human I-BABP (C) human PPARγLBD. The shaded area shows the elution position of each protein.

In a separate experiment, purified iLBP samples were applied to the HIC column in ammonium sulfate-free buffer, wherein protein absorption did not occur (data not shown). ESI-MS spectra of ethyl acetate extracts from these samples showed a protein-bound lipid profile similar to that of the non-delipidated iLBPs (data not shown). Coincidently, these iLBP samples did not display an appreciable affinity for lipid binding cavity fluorescent probes (*cf* Table 4). The HIC method can also be employed for the removal of other heterogeneous lipophilic ligands non-covalently bound to iLBPs. In our laboratory we have successfully employed this method to remove non-covalently bound ANS and lipophilic drugs to regenerate the *apo*-protein (data not shown).

In comparison, the 1-butanol liquid–liquid extraction procedure only achieved complete delipidation following three successive extractions at a 1:3 (1-butanol:protein sample) volume ratio (Table 2; Supplementary Fig. 4). Improved levels of delipidation with 1-butanol extraction could be achieved by increasing the volume of 1-butanol with respect to the volume of aqueous protein solution. However, an increase in the volume of 1-butanol concomitantly increased protein denaturation as seen from the visible protein precipitation at the aqueous–organic interface. This precipitation event was observed across all three iLBPs, data for the L-FABP sample is given as an example (Supplementary Fig. 4A). SDS-PAGE analysis of the protein aqueous phase indicated a significant loss of protein occurs with the addition of increasing 1-butanol volumes (Supplementary Fig. 4A, Table 1).

Lipidex 1000 *syn.* (HAP)-dextran type VI is a hydroxyalkylpropyl-dextran substituted approximately 10% by weight with long-chain alkyl ethers averaging 15–18 carbons in length (Supplementary Fig. 6A). Similar to the HIC method, the lipidex 1000 method achieved complete delipidation of all three test proteins following a single treatment at 37 °C (Table 2; Supplementary Fig. 5). In line with our results, Glatz and Veerkamp [16] reported that albumin can be delipidated by a single passage through a small column of lipidex 1000 at 37 °C. The effectiveness of the lipidex 1000 method is highly dependant upon temperature, there is a strict requirement to maintain the temperature at 37–45 °C throughout the procedure in order to achieve effective protein delipidation [18,34]. When operated at room temperature, lipidex 1000 failed to fully delipidate each test protein (*cf* Table 4). Similarly, literature reports have indicated that when performed at lower temperatures such as 4 °C, lipidex 1000 only removes unbound lipids whereas protein bound lipids remain [18,34]. This procedure is not suitable for the delipidation of PPARγLBD as incubation at 37 °C for prolonged times resulted in the formation of an insoluble protein precipitate, with significant losses

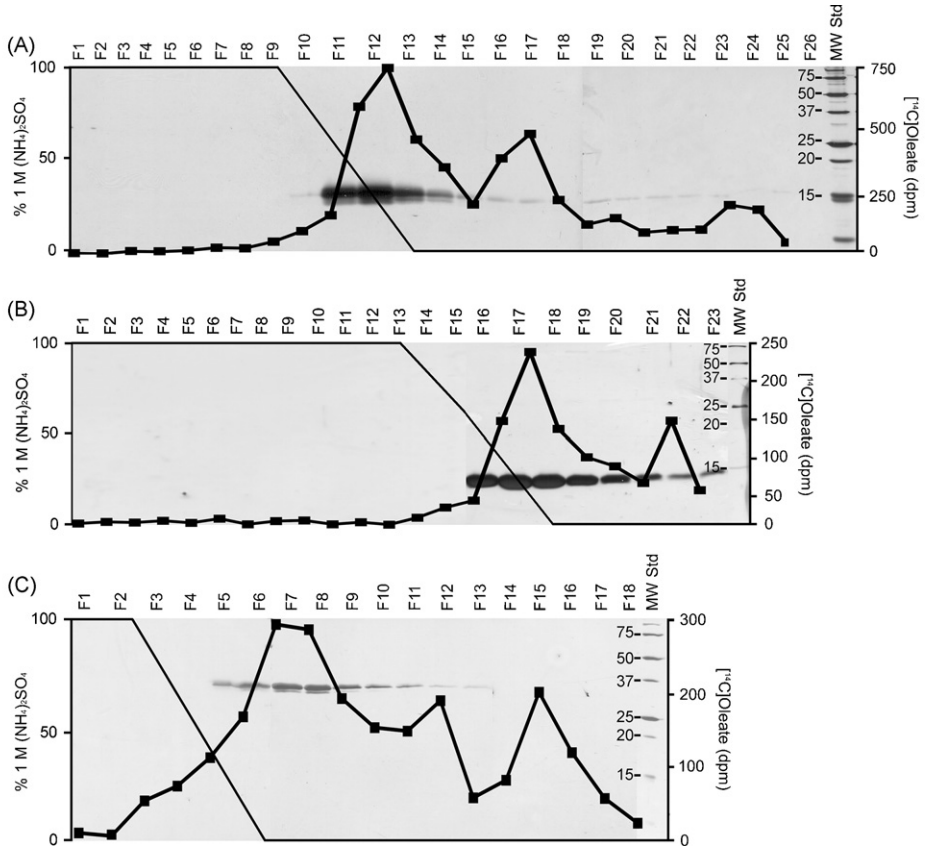

Fig. 3. HIC delipidation profile of recombinant iLBPs. The extent of protein delipidation were monitored by tracking the protein elution profile by SDS-PAGE against the counts of [^{14}C]-Oleate released with each fraction (■). The solid line represents the percentage of 1 M $(NH_4)_2SO_4$. (A) Human L-FABP (B) human I-BABP (C) human PPARγLBD.

Table 2
Quantitative assessment of the degree of delipidation of recombinant iLBPs

	% [^{14}C]-Oleate radioactivity					
Protein	HIC delipidation		Lipidex 1000 delipidation at 37 °C		1-Butanol delipidation X3 extractions	
	% Protein bound	% Recovery of total	% Protein bound	% Recovery of total	% Protein bound	% Recovery of total
L-FABP	0.43 ± 0.1	81.5 ± 7.8	0.70 ± 0.3	84.6 ± 8.2	0.81 ± 0.3	93.2 ± 7.8
I-BABP	0.19 ± 0.08	74.6 ± 12.9	0.4 ± 0.2	88.0 ± 10.1	0.3 ± 0.09	96.1 ± 6.2
PPARγLBD	0.26 ± 0.08	83.9 ± 6.2	0.8 ± 0.4	89.7 ± 9.6	0.6 ± 0.3	96.7 ± 8.3

Mean values from three independent experiments are indicated ± standard deviation.

of the starting soluble PPARγLBD protein (Table 1). Thermal stability analysis by differential scanning calorimetry indicated PPARγLBD begins to unfold at 36 °C (Fig. 4A). I-BABP is also a temperature sensitive protein that starts to unfold at 48 °C (Fig. 4B), in comparison L-FABP is very temperature stable and only starts to unfold at 65 °C (Fig. 4C). The calorimetric thermal transition values, transition temperature mid-point (t_m), enthalpy (ΔH_{cal}) and entropy (ΔS_{cal}) are documented in Table 3.

The delipidation efficacy of Sephadex LH-20 (hydroxpropyl-dextran that is not derivatized with C_{15}–C_{18} alkyl chains, Supplementary Fig. 6B) was also examined under the same buffer and temperature conditions. Complete delipidation could not be achieved even following three successive treatments with Sephadex LH-20 (data not shown). Therefore, the long-chain alkyl ether substituents appear to be the critical property of the (HAP)-dextran gel matrix required for lipid absorption.

Table 3
The thermodynamic stability parameters of human L-FABP, I-BABP and PPARγLBD calculated from thermal transition curves (*cf* Fig. 4)

Protein	t_m (°C)	ΔH_{cal} (kcal/mol)	ΔS_{cal} (kcal/K mol)
PPARγLBD	45.5	47.5	0.15
I-BABP	56.8	42.4	0.13
L-FABP	80.1	38.6	0.11

3.3. Ligand binding measurements to access protein functionality and the extent of delipidation

In a separate series of experiments, the effectiveness of each delipidation procedure was examined by fluorometric ligand binding measurements before and after each treatment (Table 4). The ligand binding affinities of each protein were measured using the fluorescent probes ANS (L-FABP binding measurements), *cis*-parinaric acid (PPARγLBD binding measurements) and bisANS (I-BABP binding measurements), that have previously been shown to bind specifically in the lipid binding cavity of these proteins [26–28]. The binding data indicated the proteins did not have an appreciable affinity for the fluorescent probes before delipidation, again suggesting endogenous lipids remain protein-bound

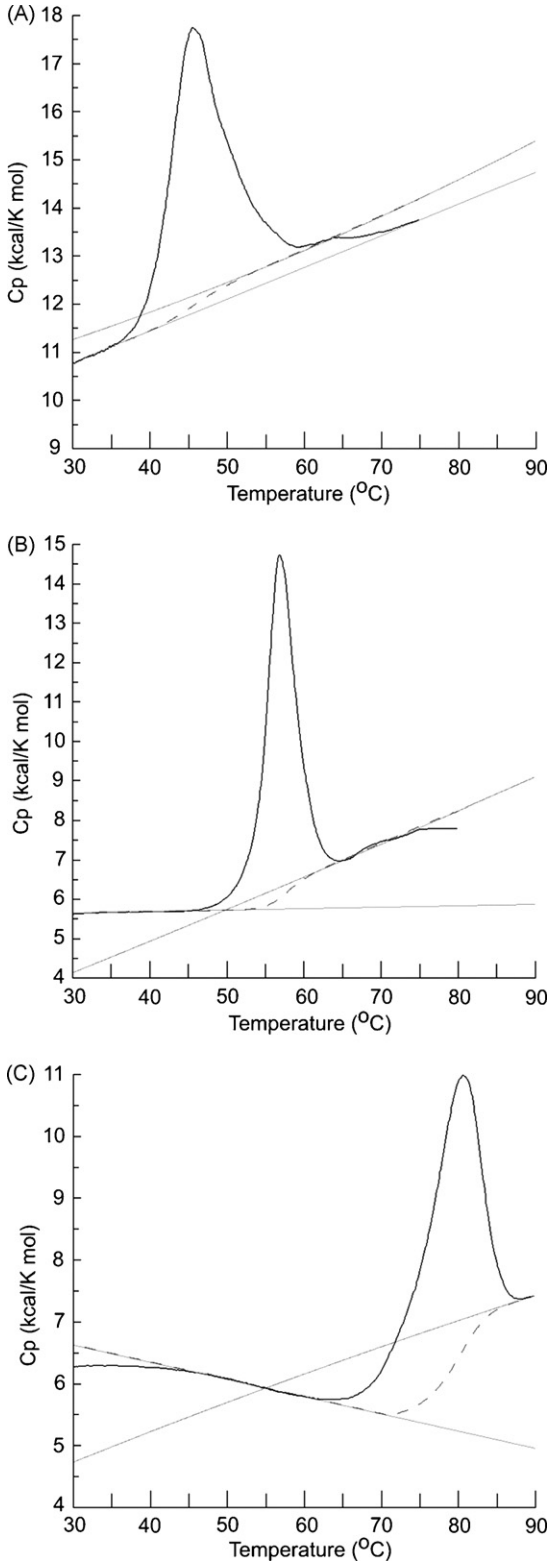

Fig. 4. The thermal denaturation of human (A) PPARγLBD, (B) I-BABP and (C) L-FABP, were monitored by DSC as described under materials and methods. Data are shown as excess specific heat capacity (Cp, kcal/K mol; dark grey curves). The pre- and post-transition base-line data fits are shown as light grey lines.

Table 4
Binding affinity values of fluorescent binding site probes for purified lipid binding proteins, before and after delipidation treatments

Protein	K_d						
	Not delipidated	HIC delipidation with $(NH_4)_2SO_4$ promoted protein absorption	HIC delipidation without protein absorption	1-Butanol delipidation	Lipidex 1000 delipidation at 37 °C	Lipidex 1000 delipidation at 20 °C	Sephadex LH-20 delipidation
L-FABP	ND[a]	K_{d1} 0.84 ± 0.3 μM[b] K_{d2} 36.3 ± 6.8 μM[b]	ND[a]	K_{d1} 1.5 ± 0.6 μM[b] K_{d2} 45.1 ± 5.8 μM[b]	K_{d1} 1.0 ± 0.4 μM[b] K_{d2} 50.4 ± 5.7 μM[b]	K_{d1} 8.5 ± 3.0 μM[b] K_{d2} 160 ± 10 μM[b]	ND[a]
I-BABP	ND[a]	6.1 ± 2.3 μM[c]	ND[a]	5.5 ± 1.2 μM[c]	6.3 ± 1.5 μM[c]	30*7.1 mM	ND[a]
PPARγLBD	4.9 ± 2.1 μM[d]	0.4 ± 0.06 μM[d]	5.3 ± 1.7 μM[d]	2.5 ± 0.6 μM[d]	1.4 ± 0.2 μM[d]	4.6 ± 2.0 μM[d]	5.4 ± 1.3 μM[d]
PPARγLBD GW1929 binding affinity (K_i)	473 ± 37.6 nM	78.7 ± 21.7 nM	509 ± 56.2 nM	327 ± 85.8 nM	260 ± 54.0 nM	450 ± 33.6 nM	521 ± 66.0 nM

[a] ND, no binding detectable.
[b] ANS as the binding cavity probe; K_{d1} high affinity binding site; K_{d2} low affinity binding site.
[c] bisANS as the binding cavity probe.
[d] *cis*-Parinaric acid as the binding cavity probe.

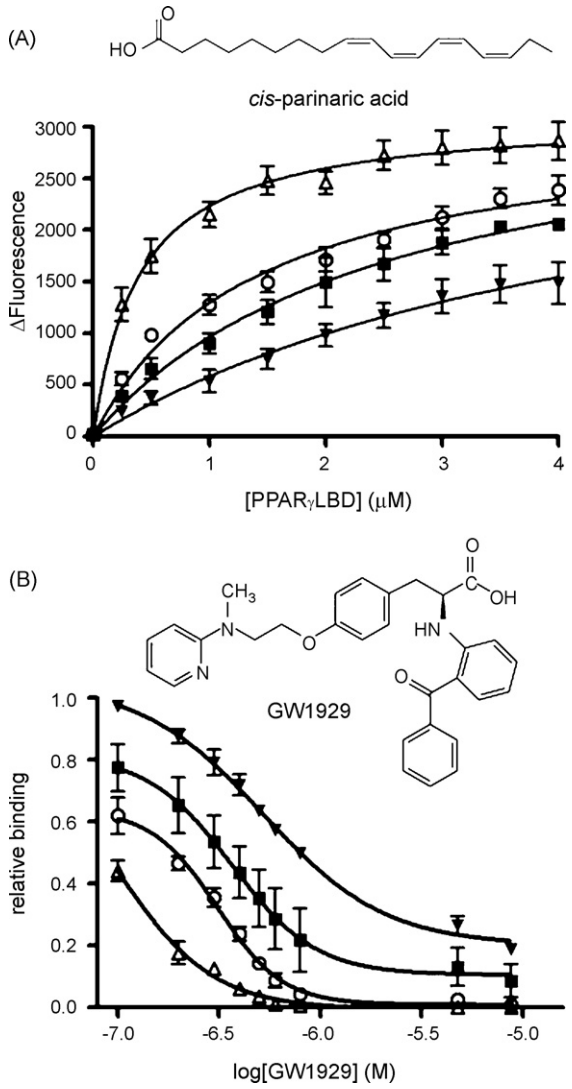

Fig. 5. (A) Binding curves for the interaction of *cis*-parinaric acid with PPARγLBD. The fluorescence increase upon titration of *cis*-parinaric acid (300 nM) with increasing concentrations of purified PPARγLBD. Dissociation constants (K_d) were determined by non-linear regression analysis of the data using a one-site binding model. (▼) not delipidated PPARγLBD; (△) HIC delipidated PPARγLBD; (○) lipidex 1000 delipidated PPARγLBD; (■) 1-butanol delipidated PPARγLBD. (B) Competition experiments. PPARγLBD (4 μM) samples from each delipidation treatment were pre-incubated with *cis*-parinaric acid (300 nM) and titrated with increasing amounts of competing ligand (GW1929) and the decrease in fluorescence was recorded. Fluorescence was normalized as a relative binding, with no-competition (relative binding = 1) and full competition (relative binding = 0). Data were analyzed by non-linear regression using a one-site competition model for the determination of K_i values for GW1929 binding affinity.

through-out the purification process. The highest binding affinities were measurable post-delipidation. The *cis*-parinaric acid binding affinity measurements with PPARγLBD, clearly demonstrate material that has been delipidated by the HIC method is more functional compared to that delipidated by the 1-butanol or lipidex 1000 methods (Fig. 5A; Table 4). The functionality of PPARγLBD following each delipidation treatment was further examined by competition experiments with the synthetic agonist GW1929 that has previously been shown to bind with high selectivity to the binding cavity of PPARγLBD [29]. The binding of GW1929 is assayed by monitoring the decrease in *cis*-parinaric acid fluorescence upon its competitive displacement from the binding cavity by titration with GW1929. The displacement data clearly demonstrates PPARγLBD delipidated by the lipidex 1000 and 1-butanol methods is less functional compared to samples treated by HIC (Fig. 5B; Table 4). Therefore, the HIC method is advantageous for the delipidation of sensitive proteins such as PPARγLBD in comparison to other methods that operate under harsher conditions.

In the case of the more robust L-FABP and I-BABP proteins, all three delipidation treatments yielded protein of comparable functionality (Table 4), thus apart from the co-sub-fractionation and higher protein yields, HIC is no better than the lipidex 1000 or 1-butanol methods for the recovery of functional L-FABP and I-BABP protein. Overall, the HIC method is only superior in terms of the recovery of more functional PPARγLBD protein.

In comparison, purified protein samples treated by Sephadex LH-20, lipidex 1000 at room temperature or HIC without ammonium sulfate promoted absorption onto the phenyl sepharose resin, did not display an appreciable binding affinity (Table 4), indicating lipid material remained non-covalently bound. These data are in line with the mass spectrometry results.

3.4. Putative mechanism of HIC delipidation

Phenyl sepharose HP is a highly cross-linked 6% agarose substituted with phenyl ligands coupled via ether linkages at a ratio of approximately 25 μmol phenyl groups/ml gel (Supplementary Fig. 6C). Although similar to reverse phase chromatography, HIC exploits the hydrophobic surface properties of proteins while operating under non-denaturing conditions. The non-polar surface area of a protein molecule can be as much as 40–50% [35]. The binding of these hydrophobic patches to the HIC absorbent is enhanced by high concentrations of anti-chaotrophic salts, and elution of the bound protein is achieved by washing with salt free buffer [36–38]. Anti-chaotrophic salts such as ammonium sulfate also have a stabilizing effect on protein structure. The mode of interaction between hydrophobic patches on the protein's surface and hydrophobic ligands immobilized on the gel matrix is believed to result from a thermodynamically favorable salting-out effect [37–39]. The driving force is an increase in entropy that produces a negative change in free energy [37]. The increase in entropy results from the displacement of ordered water molecules surrounding the immobilized hydrophobic ligands and the solvent accessible non-polar groups on the protein's surface when the two surfaces interact. The strength of this salt enhanced interaction can be estimated from the relationship between HIC retention data and salt precipitation data for the protein [40]. Thus, the chromatographic parameters that lead to protein absorption need to be optimized on a case-to-case basis. In the case of PPARγLBD, 1.0 M ammonium sulfate serves as a suitable absorption buffer whereas 3.0 M ammonium sulfate is used for L-FABP and I-BABP.

The mode of HIC delipidation presumably involves preferential absorption of lipid onto the HIC absorbent, once the protein-bound lipids exchange into the bulk solution. In the *holo*-FA complex, many FABPs form ionic interactions with the carboxyl moiety of the bound FA via the side chain of a conserved arginine residue within the binding cavity [41]. Furthermore, *in vitro* ligand binding studies have demonstrated that FABP-FA binding interactions are weakened by high salt conditions [42,43]. In light of these findings, it would be plausible to assume the high salt conditions employed in the HIC procedure would weaken this integral interaction and thereby increase the exchange rate of protein bound FA into the bulk solution. Therefore, the transient immobilization of the protein onto the HIC absorbent, coupled with the high salt solvent conditions, would promote exchange

of lipid into the bulk solution, where it preferentially re-absorbs onto the strongly hydrophobic HIC absorbent. The basis of this postulate is the observation that when applied in ammonium sulfate-free buffer, the proteins did not bind to the HIC absorbent, and delipidation was not achieved as the protein–lipid complex eluted in the void volume. Moreover, the radioactive FA tracer experiments demonstrate almost all of the applied lipid material remains bound to the HIC support following elution of the iLBP.

3.5. Summary

This report presents a simple non-destructive method that effectively removes all lipid material such as FA and steroids from aqueous proteins using HIC. In addition to serving as a delipidation process, HIC concomitantly acts as a protein purification step. HIC is very effective as an initial product capture step to separate the target protein from the bulk of the soluble contaminants. In terms of practicality, HIC is complementary to ion exchange, each technique providing separation according to hydrophobicity and charge, respectively. This approach was successfully employed for the I-BABP purification scheme. The main advantages of the HIC method compared to the lipidex 1000 or organic solvent-based delipidation methods are increased capacity, speed and protein recovery, as this process operates under fast protein liquid chromatography conditions at room temperature and serves as a concomitant protein sub-fractionation step. The HIC procedure described herein, results in complete, efficient and reproducible delipidation of aqueous lipid binding proteins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2008.04.011.

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