Journal of Chromatography B, 879 (2011) 2935–2940

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Straightforward isolation of phosphatidyl-ethanolamine-binding protein-1 (PEBP-1) and ubiquitin from bovine testis by hydrophobic-interaction chromatography (HIC)

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ARTICLE INFO

Article history:
Received 1 June 2011
Accepted 21 August 2011
Available online 26 August 2011

Keywords:
Phosphatidyl-ethanolamine-binding protein
Hydrophobic interaction chromatography
Raf-1 kinase inhibitory protein
Hippocampal cholinergic neurostimulating peptide precursor protein
Isoform

ABSTRACT

Isolation of phosphatidyl-ethanolamine-binding protein-1 (PEBP-1) from bovine brain was described almost three decades ago but it required a large number of steps to reach high purity. After the fractionation of bovine testis proteins by ammonium sulfate precipitation we found that PEBP-1, detected by Western blotting, was among the very few proteins still soluble at 80% ammonium sulfate saturation (3.2 M). This soluble fraction (S80) was directly loaded onto a phenyl sepharose column equilibrated at the same ammonium sulfate concentration (3.2 M). A stepwise elution of the retained material at 1.0, 0.5, 0.2, 0.1 M ammonium sulfate in ammonium hydrogen carbonate was performed and then with ammonium hydrogen carbonate alone and finally with 50% ethylene glycol. All fractions were analyzed by SDS–PAGE and Western blotting and the fractions containing PEBP-1 was further fractionated by size exclusion chromatography on a HR75 Superdex column permitting the isolation of ubiquitin in addition to PEBP-1 as demonstrated by Western blotting and mass spectrometry. This study shows the feasibility of hydrophobic interaction chromatography (HIC) on phenyl sepharose at a very high ammonium sulfate concentration (3.2 M; 80% saturation) to efficiently purify the proteins that are still soluble in these extreme conditions.

1. Introduction

The PEBP (phosphatidyl-ethanolamine-binding protein) family is a highly conserved group of proteins that have been identified in numerous tissues in most organisms, including bacteria, yeasts, plants, invertebrates and vertebrates. Phosphatidyl-ethanolamine-binding protein-1 (PEBP-1) is a multifunctional 21–23 kDa protein that was initially described as a cytoplasmic protein [1] but was later found associated with plasma and endoplasmic reticulum membranes in numerous organs [2] and it was also found to be secreted [3] despite its lack of a signal sequence. PEBP-1 is known under various other names corresponding to its different biological activities recognized so far in the animal kingdom: Raf-1 kinase inhibitory protein (RKIP) [4], decapacitation factor-receptor (DF-R) [5], hippocampal cholinergic neurostimulating peptide precursor protein (HCNPpp) [3,6] or serine-protease inhibitor (SPI) [7]. In addition, PEBP has been shown to regulate cell migration [8–10], to be an inhibitor of non-apoptotic programmed cell death [11] and to act as a tumor suppressor. Indeed, its expression is down-regulated in tumor interstitial fluid from breast cancer patients [12] and it negatively regulates both the MAPK and NFκB pathways that are found hyperactivated in melanomas [13]. Concerning the central nervous system, PEBP-1 has been found down-regulated in the Alzheimer disease model mouse line Tg2576 [14] but, in contrast, it is up-regulated in rat hippocampal progenitor cells which are important for memory formation [15] as well as in the brain of atlantic cod exposed to methylmercury [16].

The isolation of PEBP-1 in a highly purified state was described almost three decades ago but it required a large number of steps [1]. In the present paper, we report a straightforward method allowing the isolation of PEBP-1 by taking advantage of its high solubility in 80% saturation ammonium sulfate and the development of hydrophobic interaction chromatography on a phenyl-sepharose column equilibrated at this very high ammonium sulfate concentration. Preliminary characterization of PEBP-1 purified by this method is presented.

2. Materials and methods

Bovine testes were obtained at a local slaughterhouse immediately after sacrifice of the animals (3–6 months of age). Chemicals were of the highest grade available and were obtained from Sigma

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doi:10.1016/j.jchromb.2011.08.028

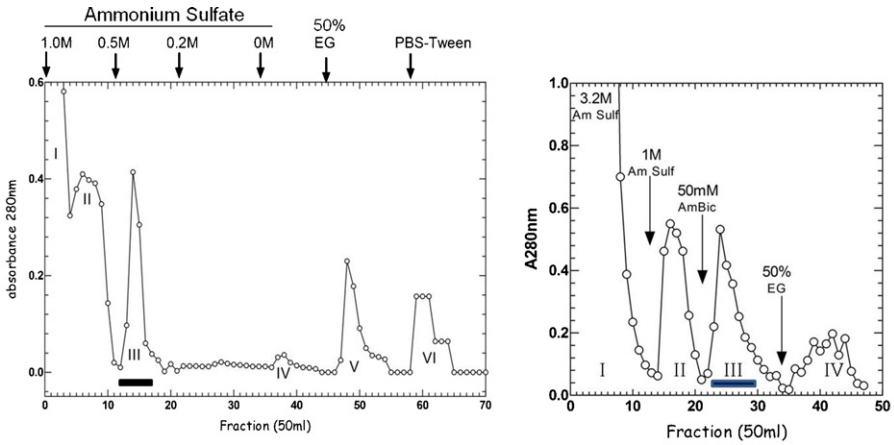

Fig. 1. Hydrophobic Interaction Chromatography of the soluble fraction after 80% ammonium sulfate saturation precipitation of the proteins extracted from bovine testes. The column was equilibrated in 3.2 M ammonium sulfate (~80% saturation) before loading of the S80 fraction (soluble fraction after 80% ammonium sulfate saturation precipitation). After total loading, the column was first eluted with 1 M ammonium sulfate. It was then eluted with 0.5 M and 0.2 M ammonium sulfate then with 0.05 M ammonium hydrogen carbonate, 50% (v/v) ethylene glycol in 0.05 M ammonium hydrogen carbonate and finally with Tween in PBS (left) or it was directly eluted with 0.05 M ammonium hydrogen carbonate and finally with 50% (v/v) ethylene glycol in 0.05 M ammonium hydrogen carbonate (right).

(St-Quentin-Fallavier, France) unless otherwise stated. Phenyl-sepharose gel as well as Sephadex G25 and Superdex HR75 columns were from GE Healthcare France. Dialysis membrane (cut off 12-14000) was from Medicell Intl Ltd (London, UK).

The phenyl-sepharose column (5 cm × 12 cm) was equilibrated in 3.2 M ammonium sulfate before use and elution steps were performed at 3.2, 1.0, 0.5, 0.2, 0.1 and 0 M in 50 mM ammonium hydrogen carbonate followed by washing with 50% ethylene glycol. In an alternative protocol, elution steps were performed at 3.2, 1.0, and then directly 0 M in 50 mM ammonium hydrogen carbonate.

Small-scale preparative HPLC was performed on a Superdex 75 column using a GE Healthcare Ettan apparatus at a flow rate of 200 μl/min using 50 mM ammonium hydrogen carbonate for elution. The fractions were recovered, freeze-dried and subsequently analyzed.

Mass spectrometric identification of the protein was performed by Ms Lucie Spina at the MassSpec laboratory (PAIB) in our Institute using an ESI-TRAP linear ion-trap spectrometer (Thermo Electron Co, Waltham, Mass, USA) and the Mascot identification program [17]. Mass determination of purified materials was carried out by MALDI-TOF.

Recombinant bovine PEBP expressed in *E. coli* [18,19] was kindly provided by Dr. Françoise Schoentgen (CNRS, IMPMC, Paris, France). The anti-PEBP IgYs were obtained from chicken eggs and purified by affinity chromatography (Interchim, Montluçon, France) whereas rabbit anti-ubiquitin polyclonal antibodies were previously characterized [20].

For SDS–PAGE and Western blot analyses, an aliquot of each fraction was diluted in Laemmli's 4× buffer under nonreducing or reducing conditions [21]. Samples were heated or not for 10 min at 100 °C and electrophoresed in 15% SDS–PAGE. Gels were either silver or coomassie blue stained or transferred overnight at 4 °C onto nitrocellulose membranes (Schleicher and Schuell, Ecquevilly, France). The membranes were probed with the anti-PEBP antibody (1:2500, 4 h at room temperature), then one hour at room temperature with 1:100,000 donkey peroxidase-conjugated anti-chicken antibodies (Jackson ImmunoResearch, Baltimore, PA, USA). Membranes were visualized using the ECL™ Western Blotting Detection Reagents (Amersham Biosciences Orsay, France) after a 15-min exposure.

Hydroxylamine treatment (1 M pH 7.0 at 30 °C for 16 h) was used to specifically hydrolyze putative thioester linkage between cysteine residue and palmitic acid chain [22,23].

3. Results

3.1. Purification

The testes were rapidly brought back to the laboratory in melting ice and after elimination of the albuginae were cut into small dices. This material was then extracted in 1 l of 0.15 M ammonium sulfate per kg tissue by vigorous grinding using an Ultra-Turax (Janke & Kunkel, Staufen, Germany) at full speed at 4 °C. After centrifugation (5000 × *g*, 30 min), the supernatant was adjusted to 1 M ammonium sulfate (approximately 25% saturation at 4 °C) by addition of the solid salt under stirring and kept overnight at 4 °C before centrifugation in the same conditions as above. The resulting supernatant was then added with solid ammonium sulfate up to 50% saturation (2.0 M) at 4 °C and kept overnight at this temperature. After centrifugation, the supernatant was recovered and added with solid ammonium sulfate up to 80% saturation (3.2 M) at 4 °C. After overnight precipitation and centrifugation, the final supernatant (S80) was recovered and loaded on a phenyl-sepharose column (5 cm × 12 cm) equilibrated in 3.2 M ammonium sulfate elution buffer. Stepwise elution was then performed at 1.0, 0.5, 0.2 and 0.1 M ammonium sulfate in 50 mM ammonium hydrogen carbonate and then with 50 mM ammonium hydrogen carbonate alone and finally with 50% ethylene glycol (Fig. 1, left panel).

PEBP-1 was mainly found in the 0.5 M ammonium sulfate fraction (fraction III). This fraction was then dialyzed against demineralized water at 4 °C, freeze-dried and submitted to desalting chromatography on a Sephadex G25 column equilibrated in 50 mM ammonium hydrogen carbonate.

Since almost no material eluted at 0.2 M and 0.1 M ammonium sulfate, the standard elution scheme was modified as to eluate the PEBP-containing fraction directly with 50 mM ammonium hydrogen carbonate after the 3.2 M and 1 M ammonium sulfate steps (Fig. 1, right panel) in the following purifications. As expected, PEBP was eluted in fraction III in the absence of ammonium sulfate.

The analysis of fraction III by 2D-SDS–PAGE (Fig. 2) showed that it contained only a few proteins, with PEBP as the main component and ubiquitin as a minor component as determined by specific Western blotting.

Size-exclusion chromatography on Superdex HR75 column was chosen as a final step for PEBP (Fig. 3). PEBP was detected in fraction 3 by Western blotting (Fig. 3B) and its elution volume as well

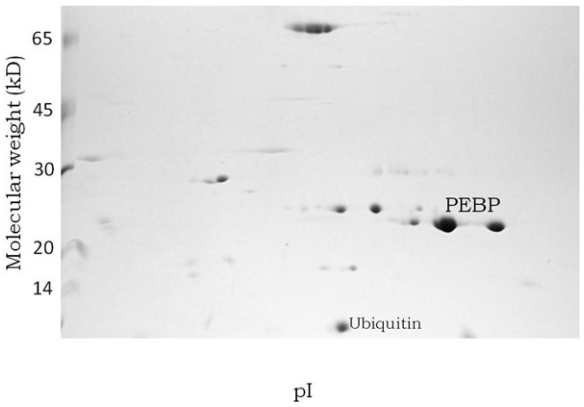

Fig. 2. 2D-SDS–PAGE of fraction III from Hydrophobic Interaction Chromatography (Fig. 1, right). PEBP and ubiquitin were identified by Western blotting (Fig. 3) and mass spectrometry (Fig. 5).

as its mobility in SDS–PAGE were in agreement with its expected molecular weight. In parallel, the 8500 Da protein which was purified in peak 4 was identified as ubiquitin by Western blotting (Fig. 3C).

3.2. Characterization

The purified bovine testis PEBP was compared to recombinant bovine PEBP expressed in *E. coli* in a SDS–PAGE and Western-blotting. Fig. 4 shows that the testicular 21 kDa protein exhibits the same mobility as authentic recombinant PEBP in SDS–PAGE and that both are specifically recognized by anti-PEBP IgYs. In non-reducing conditions two bands were evidenced with the minor one exhibiting a slightly lower mobility. The two bands were detected by Western blotting indicating that the two molecules are two forms of PEBP. In reducing conditions, only one band was detected and it was also readily detected in Western blotting.

Mass spectrometry analysis of the 21 kDa protein recovered from the size-exclusion chromatography on HR75 (Fig. 3, fraction 3) confirmed that this protein is PEBP. Indeed Fig. 5 shows a major peak at 20853 that is fully consistent with the calculated molecular mass of PEBP (20881). A minor peak was also detected that exhibits a mass of 21,093 that is thus about 212 Da higher than the major peak.

These two peaks detected by mass spectrometry correspond to the two bands evidenced by SDS–PAGE and Western blotting. In reductive conditions (mercaptoethanol at 100 °C), the higher-molecular weight band disappeared, suggesting that it is transformed into the lighter one. In order to test the hypothesis

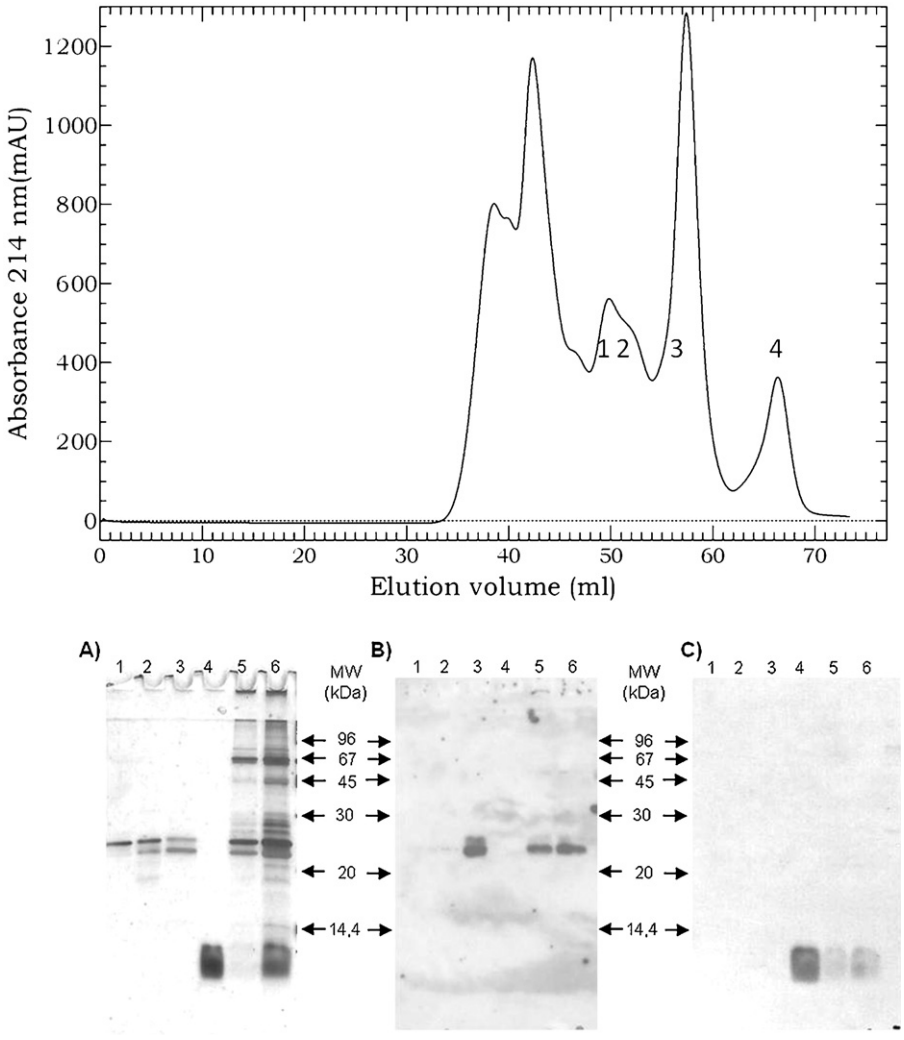

Fig. 3. (Upper panel) Size-exclusion chromatography of fraction III from hydrophobic interaction chromatography (Fig. 1, right) on Superdex HR 75. The figures show the fractions analyzed by SDS–PAGE and Western blotting. (Lower panel) SDS–PAGE (15%) and Western-blottings of 2 μg of fractions 1–4 from Superdex HR75 chromatography above and of 5 and 10 μg starting material (5, 6). A: silver staining; B: Western blotting with anti-PEBP; C: Western blotting with anti-ubiquitin.

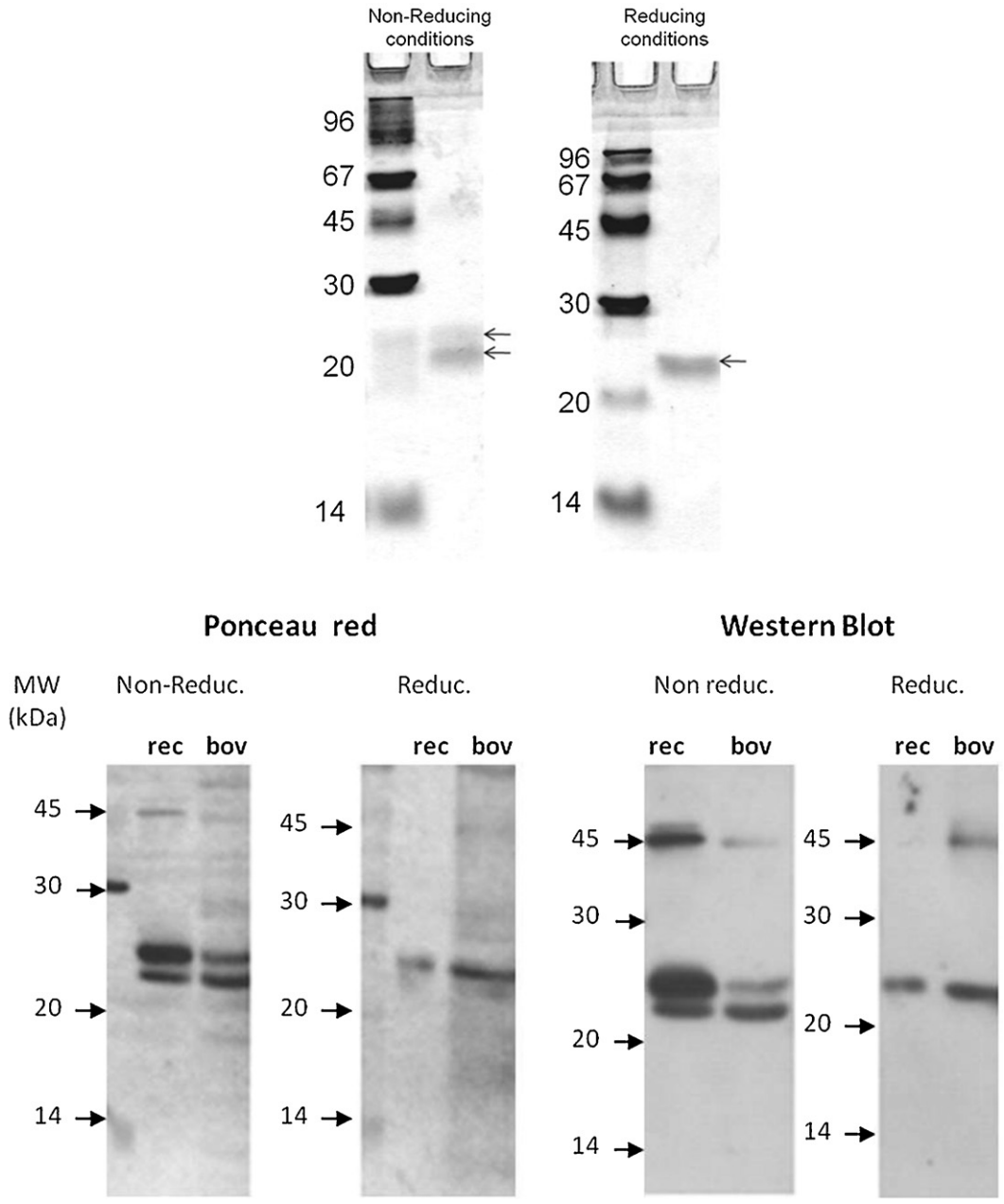

Fig. 4. Comparison of bovine PEBP with recombinant PEBP expressed in *E. coli* and effect of mercaptoethanol reduction. (Upper panel) SDS–PAGE and coomassie blue staining. (Lower panel) SDS–PAGE and ponceau red staining (left) and Western blotting (right).

that the ~212 Da adduct is due to S-palmitoylation of one cysteine residue in PEBP-1, the preparation was submitted to 1 M hydroxylamine treatment at 30 °C in order to specifically cleave the putative thiolester linkage. No modification in the ratio of the two PEBP bands in SDS–PAGE was observed after this treatment.

The protein eluted in fraction 4 in size-exclusion chromatography on Superdex-75 (Fig. 3) was shown to exhibit a molecular mass of 8450 (Fig. 5B) consistent with that of ubiquitin (calc. MW 8434) and confirmed by Western blotting data (Fig. 3C).

4. Discussion

Phosphatidyl-ethanolamine-binding protein (PEBP) has been shown to possess a large number of functions leading to its description under various names: Raf-1 kinase inhibitory protein (RKIP) [4], decapacitation factor-receptor (DF-R) [5] or hippocampal cholinergic neurostimulating peptide precursor protein (HCNPpp) [3,6]. Concerning intracellular signaling pathways, in addition to its RKIP activity, PEBP has been found to interact with G protein-dependent signaling through its interaction with GRK-2 [24] and to inhibit four kinases of the NFκB pathway [25]. It has also been shown that PEPB is phosphorylated by protein kinase C (PKC) and, in consequence, 1/it interacts with, and inhibits the activity of GRKs on G protein-coupled receptors (GPCR) [26] and 2/it exhibits a diminished RKIP activity [27]. Therefore, PKC-catalyzed PEBP phosphorylation increases both the phosphoinositide and MAPK pathways [28]. PEBP has also been shown to inhibit the activity of various proteases such as thrombin or neuropsin but not chymotrypsin, plasminogen activator or elastase [7]. Recently, it has also been shown to play a role in cell migration and to be the target of locostatin, a small-molecule inhibitor of cell movement [8,9]. In view of this large spectrum of intra- and extra-cellular

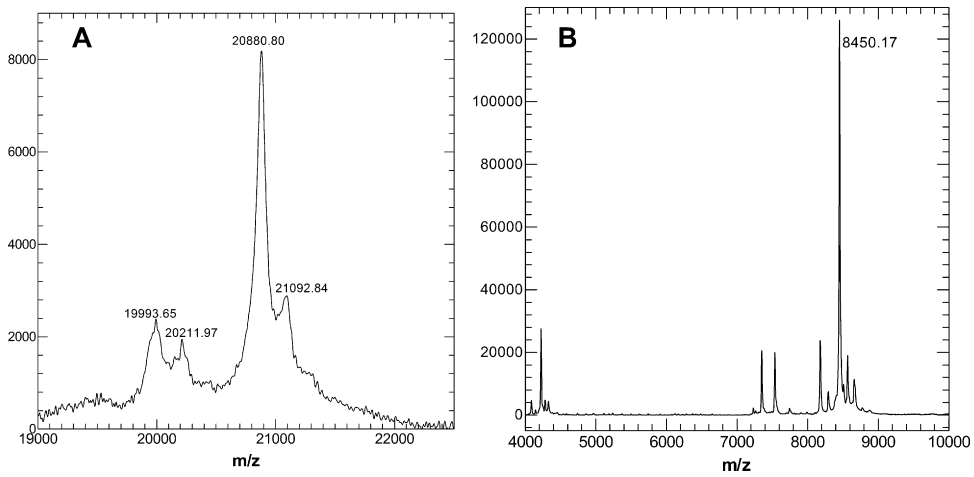

Fig. 5. (A) MALDI-TOF of fraction 3 from size-exclusion chromatography on Superdex HR 75 (Fig. 3). (B) MALDI-TOF of fraction 4 from size-exclusion chromatography on Superdex HR 75 (Fig. 3).

functions, it is of utmost interest to obtain this molecule in high amount in purified form in order to study its structure–functions relationships.

Isolation [1] and characterization [29–33] of bovine brain PEBP were performed a long time ago but the isolation of large quantities of the protein in a highly purified form appeared very cumbersome. We thus developed a more straightforward procedure for its purification.

The classical ammonium sulfate precipitation technique was found particularly efficient for PEBP isolation as the protein was one of the few ones remaining soluble at 80% ammonium sulfate saturation. Rather than getting rid of ammonium sulfate by dialysis we tested if phenyl-sepharose could be used at 80% ammonium sulfate saturation and if the protein could be retained in these conditions and eluted by diminishing ammonium sulfate concentration. PEBP was indeed retained and it was mainly found in the 0.5 M ammonium sulfate fraction during HIC stepwise elution. Not only HIC permits separation of PEBP from most of its contaminants but it is also a convenient step to concentrate the proteins from the bulky S80 fraction. Size-exclusion chromatography was found to be efficient as the final step of PEBP isolation (Fig. 3).

Two close PEBP bands were detected by SDS–PAGE and Western-blotting in non-reducing conditions If one band is obviously expected to be authentic PEBP, the second one can be either slightly heavier or slightly lighter than it. It is important to notice that only one band is detected in SDS–PAGE in reducing conditions indicating that this second PEBP-1 form is not due to a longer or shorter amino-acid sequence.

Mass spectrometry data clearly indicate that the most abundant form is authentic PEBP-1 and that the minor form exhibits a molecular mass that is approximately 212 Da higher. Since the two forms were still detected in SDS–PAGE even after heating at 100 °C for 10 min, the form with the higher molecular weight is not simply PEBP-1 with a ligand but is rather due to a covalent post-translational adduct. The molecular weight of this conjugate (~212 Da) led us to hypothesize that the high-molecular weight PEBP-1 might possess a S-thioester palmitoyl chain. This modification which is known to be dynamic would explain the presence of PEBP-1 in cytoplasmic as well as in membrane compartments of the cell. However, concentrated hydroxylamine treatment did not affect the ratio of the two isoforms thus strongly arguing against this hypothesis. Another opposing argument is the fact that the same two isoforms are detected in recombinant PEBP-1 expressed in *E. coli* (Fig. 4). Since bacteria are not known to possess S-palmitoyl transferase, it is very likely that the covalent modification leading to the second isoform is not S-palmitoylation.

PEBP is known to be phosphorylated by protein kinase C [28] but it is unlikely that the second isoform is phosphorylated PEBP as it disappears upon reduction with mercaptoethanol. Nevertheless it cannot be ruled out that non-phosphorylated and phophorylated PEBPs exhibit similar mobilities in reductive conditions. If so, the second isoform might be phophorylated PEBP but this remains to be checked.

The ubiquitary protein PEBP exhibits a considerable variety of functions that have been previously described. As it is a small protein and does not possess various domains, it can be expected that it plays these diverse roles through a common physico-chemical property. The isolation of natural PEBP isoforms from various sources is thus essential to decipher their structure-function relationships. The straightforward isolation process described here should contribute to the achievement of this goal.

5. Conclusion

The availability of PEBP in large amounts using this straightforward isolation technique will allow to more thoroughly study the very diverse functions of PEBP-1 in relation with its structure. Moreover, as the procedure described in this paper is simple and convenient for the purification of PEBP-1 from bovine testes, it can most probably be used for the isolation of PEBP-1 from other tissues and from other species as well. Finally, the feasibility of hydrophobic interaction chromatography on phenyl-sepharose at much higher ammonium sulfate concentration than that recommended by the manufacturer has also been shown efficient for ubiquitin purification and could be of interest for other highly soluble proteins as well.

Acknowledgements

We are indebted to Dr Françoise Schoentgen (CNRS, IMPMC, Paris, France) for her generous gift of recombinant bovine PEBP produced in *E. coli*, to Mr Guillaume Tsikis (INRA-CNRS PRC, Nouzilly, France) for performing 2D-SDS–PAGE, to Pascal Papillier and Jean-Noël Couët (INRA-CNRS PRC, Nouzilly, France) for collecting the organs very early in the morning at the slaughterhouse and to Lucie Spina and Valérie Labas (Laboratoire de Spectrométrie de Masse du Centre de Recherches INRA de Tours, PAIB, Nouzilly, France) for mass spectrometry analyses. Thanks are due to INRA and to the

Région Centre Council (Orléans, France) for a joint PhD grant to MHH.

References

[1] I. Bernier, P. Jolles, Biochim. Biophys. Acta 790 (1984) 174.
[2] J. Frayne, C. Ingram, S. Love, L. Hall, Cell Tissue Res. 298 (1999) 415.
[3] K. Ojika, S. Mitake, N. Tohdoh, S.H. Appel, Y. Otsuka, E. Katada, N. Matsukawa, Prog. Neurobiol. 60 (2000) 37.
[4] K. Yeung, T. Seitz, S. Li, P. Janosch, B. McFerran, C. Kaiser, F. Fee, K.D. Katsanakis, D.W. Rose, H. Mischak, J.M. Sedivy, W. Kolch, Nature 401 (1999) 173.
[5] R. Gibbons, S.A. Adeoya-Osiguwa, L.R. Fraser, Reproduction 130 (2005) 497.
[6] K. Ojika, S. Kojima, Y. Ueki, N. Fukushima, K. Hayashi, M. Yamamoto, Brain Res. 572 (1992) 164.
[7] U. Hengst, H. Albrecht, D. Hess, D. Monard, J. Biol. Chem. 276 (2001) 535.
[8] J. Ma, F. Li, L. Liu, D. Cui, X. Wu, X. Jiang, H. Jiang, Liver Int. 29 (2009) 567.
[9] W.M. Bement, Chem. Biol. 12 (2005) 953.
[10] A.N. Shemon, E.M. Eves, M.C. Clark, G. Heil, A. Granovsky, L. Zeng, A. Imamoto, S. Koide, M.R. Rosner, PLoS One 4 (2009) e6028.
[11] S. Sperandio, K.S. Poksay, B. Schilling, D. Crippen, B.W. Gibson, D.E. Bredesen, J. Cell. Biochem. 111 (2010) 1401.
[12] L. Cortesi, A. Barchetti, E. De Matteis, E. Rossi, L. Della Casa, L. Marcheselli, G. Tazzioli, M.G. Lazzaretti, G. Ficarra, M. Federico, A. Iannone, J. Proteome Res. 8 (2009) 4916.
[13] K. Lin, S. Baritaki, L. Militello, G. Malaponte, Y. Bevelacqua, B. Bonavida, Genes Cancer 1 (2010) 409.
[14] A.J. George, R.M. Holsinger, C.A. McLean, S.S. Tan, H.S. Scott, T. Cardamone, R. Cappai, C.L. Masters, Q.X. Li, Neurobiol. Aging 27 (2006) 614.
[15] T. Sagisaka, N. Matsukawa, T. Toyoda, N. Uematsu, T. Kanamori, H. Wake, C.V. Borlongan, K. Ojika, Brain Res. 1327 (2010) 107.
[16] K. Berg, P. Puntervoll, S. Valdersnes, A. Goksoyr, Aquat. Toxicol. 100 (2010) 51.
[17] T. Koenig, B.H. Menze, M. Kirchner, F. Monigatti, K.C. Parker, T. Patterson, J.J. Steen, F.A. Hamprecht, H. Steen, J. Proteome Res. 7 (2008) 3708.
[18] B. Vallee, C. Teyssier, R. Maget-Dana, J. Ramstein, N. Bureaud, F. Schoentgen, Eur. J. Biochem. 266 (1999) 40.
[19] C. Atmanene, A. Laux, E. Glattard, A. Muller, F. Schoentgen, M.H. Metz-Boutigue, D. Aunis, A. Van Dorsselaer, G.B. Stefano, S. Sanglier-Cianferani, Y. Goumon, Med. Sci. Monit. 15 (2009) BR178.
[20] C. Cahoreau, L. Garnier, J. Djiane, G. Devauchelle, M. Cerutti, FEBS Lett. 350 (1994) 230.
[21] U.K. Laemmli, Nature 227 (1970) 680.
[22] P.M. Steinert, S.Y. Kim, S.I. Chung, L.N. Marekov, J. Biol. Chem. 271 (1996) 26242.
[23] R. Chakravarty, R.H. Rice, J. Biol. Chem. 264 (1989) 625.
[24] T. Kroslak, T. Koch, E. Kahl, V. Hollt, J. Biol. Chem. 276 (2001) 39772.
[25] K.C. Yeung, D.W. Rose, A.S. Dhillon, D. Yaros, M. Gustafsson, D. Chatterjee, B. McFerran, J. Wyche, W. Kolch, J.M. Sedivy, Mol. Cell. Biol. 21 (2001) 7207.
[26] K. Lorenz, M.J. Lohse, U. Quitterer, Nature 426 (2003) 574.
[27] K.C. Corbit, N. Trakul, E.M. Eves, B. Diaz, M. Marshall, M.R. Rosner, J. Biol. Chem. 278 (2003) 13061.
[28] W. Kolch, Nat. Rev. Mol. Cell Biol. 6 (2005) 827.
[29] F. Schoentgen, F. Saccoccio, J. Jolles, I. Bernier, P. Jolles, Eur. J. Biochem. 166 (1987) 333.
[30] F. Schoentgen, N. Seddiqi, S. Bucquoy, P. Jolles, L. Lemesle-Varloot, K. Provost, J.P. Mornon, Protein Eng. 5 (1992) 295.
[31] I. Bernier, J.P. Tresca, P. Jolles, Biochim. Biophys. Acta 871 (1986) 19.
[32] S. Bucquoy, P. Jolles, F. Schoentgen, Eur. J. Biochem. 225 (1994) 1203.
[33] F. Schoentgen, L.M. Bonanno, G. Pignede, P. Jolles, Mol. Cell. Biochem. 98 (1990) 35.