Characterization of human placental alkaline phosphatase by activity and protein assays, capillary electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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Abstract

Placental alkaline phosphatase (PLAP) that had been isolated from human placenta was further purified using subsequent ion-exchange chromatography (IEC), affinity chromatography (AC) and centrifugal membrane concentration (CMC). During the process, the PLAP samples from the different stages of purification were characterized regarding purity and activity. This was accomplished by combining Lowry analysis, enzymatic activity assay, capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE) and matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF-MS). The sample obtained after IEC had a rather low specific activity (6.8 U/mg) and appeared to contain several major contaminants, among which was human serum albumin (HSA). AC followed by CMC yielded PLAP with a specific activity of 128 U/mg. The purity and identity of the protein was indicated by MALDI-TOF-MS yielding a spectrum with one major peak at m/z 58 101. Interestingly, CZE of the pure PLAP revealed a cluster of peaks, which probably reflects the presence of various glycoforms and/or oligomers. The same analytical approach was used to characterize commercially available PLAP. This sample showed a moderate specific activity (15 U/mg) and appeared to be highly impure containing various other proteins. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Alkaline phosphatases (APs) are a group of cell-membrane-associated enzymes which act on a variety of phosphorylated substrates and share features such as high pH optima, dependence on magnesium and zinc ions and a dimeric structure. APs (EC 3.1.3.1) exist in humans in four different isoforms: intestinal, germ cell, tissue non-specific (liver, bone, kidney) and placental. Several estimations of the molecular mass of APs have been presented. For example, the molecular mass of the monomers of the placental form has shown to be M_r 58 000 [1] and 64 000 [2]. AP was already demonstrated in human serum in the 1920s [3] and later its relevance in various diseases was shown [4–9]. Recently it was discovered that APs can detoxify endotoxin produced by Gram-negative bacteria. Endotoxin is known to

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cause sepsis [10], and consists of lipopolysaccharides, which contain phosphate groups that are essential for the toxicity. AP can remove these groups and thus reduce the endotoxin toxicity.

Within our university the potential use of placental alkaline phosphatase (PLAP) for the treatment of sepsis is investigated. In our groups we set out to develop dosage forms of PLAP and investigate analytical methods to establish the purity and stability of PLAP. Frequently, the purity and concentration of PLAP samples is being checked only by monitoring its enzymatic activity. This method does not provide any information on the presence of inactive enzyme and other degradation products or contaminants.

Fast protein liquid chromatography (FPLC) is often used for separation of proteins, and the separation of AP forms from bovine intestine, human placenta and human liver has been reported [11]. Also, liver and bone isoenzyme of AP from human serum has been separated by applying high-performance affinity chromatography (HPAC) [7]. The detection of APs is often accomplished by post-column conversion of *p*-nitrophenyl phosphate (pNPP) into a colored product but, with this method, no information about the presence of sample constituents other than AP is obtained. With this approach APs can be detected very sensitively but, since only active AP is detected, no information about the presence of other sample constituents is obtained. In principle, good protein resolutions can be obtained with reversed-phase high-performance liquid chromatography (RP-HPLC) [12–14], but so far no application of RP-HPLC for the analysis of APs has been described.

Capillary electrophoresis (CE) shows good potential for the analysis of proteins. With CE high efficiencies can be obtained, offering the possibility to separate a protein from similar compounds such as closely related impurities and decomposition products. For example, glycoforms of ovalbumin [15–17], recombinant erythropoietin [18], fetuin and α_1-acid [19] have been separated by CE. Also, a few CE methods for the indirect quantification of APs have been described [20–22] in which substrate is enzymatically converted into a colored or fluorescent product, which subsequently is separated and detected. However, with this approach obviously only

the activity of AP is measured and no other compounds can be determined.

In this paper the necessity of comprehensive quality control will be illustrated on the basis of a purification/isolation procedure for PLAP from human placenta employing ion-exchange chromatography (IEC) and affinity chromatography. The effectiveness of the purification steps was monitored by enzymatic activity assay, Lowry analysis (protein content), capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The usefulness of the approach is also demonstrated by the analysis of a commercially available PLAP sample.

2. Experimental

2.1. Materials

Magnesium chloride, ammediol (2-amino-2-methyl-1,3-propanediol), tricine, sodium chloride, magnesium sulfate, 4-nitrophenyl phosphate (pNPP), and trifluoroacetic acid (TFA) were from Fluka (Buchs, Switzerland). Boric acid, sodium hydroxide, Folin reagent, sodium carbonate, sodium potassium tartrate, Tris (tris-[hydroxymethyl]aminomethane) and acetic acid were purchased from Merck (Darmstadt, Germany). Copper sulfate was from Genfarma (Maarssen, The Netherlands) and putrescine (1,4-diaminobutane) from Aldrich (Gillingham, UK). Ferulic acid (4-hydroxy-3-methoxycinnamic acid), sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) and human placental alkaline phosphatase (EC 3.1.3.1) were from Sigma (St. Louis, MO, USA). Water used in the experiments was from an Elga Maxima Ultra Pure Water apparatus (Salm & Kipp, Breukelen, The Netherlands). Ethanol and 2-propanol were from Merck and acetonitrile from Labscan (Dublin, Ireland). Bovine serum albumin (BSA) was from ICN Biochemicals (Aurora, OH, USA) and β-mercaptoethanol from Life Technologies (Rockville, MD, USA). For dialysis of PLAP fractions Spectra/Por dialysis tubing from Spectrum Companies (Gardena, CA, USA) was used. Centrisart-C 30 centrifuge filters with a molecular mass cut-off (MWCO) of 10 000 were from Sartorius (Göttingen,

Germany). Human placenta was kindly donated by the University Hospital Groningen, The Netherlands.

2.2. Purification

2.2.1. Extraction of human placenta

Briefly the extraction procedure of the human placenta was as follows. Human placenta (ca. 50 g) was cut into pieces and homogenized in a blender with 560 ml Tris buffer (0.5 M, pH 7.4) and 280 ml butanol. Subsequently, 280 ml butanol was added followed by rigorous stirring for 75 min. The mixture was then centrifuged (23 000 g, 4°C) for 10 min, and the butanol fraction was removed. After filtration, the water phase was dialyzed (MWCO 60 000) at 4°C against Tris buffer (0.5 M, pH 7.4) with 5 mM magnesium sulfate.

2.2.2. Ion-exchange chromatography

IEC was performed on a diethyl-aminoethyl (DEAE-52) column (45 cm×2.5 cm I.D.) using an Econo pump, an Econo UV monitor and a Model 2110 fraction collector from Bio-Rad (Hercules, CA, USA). Extracts were loaded onto the column, which then was flushed at 1 ml/min using a buffer of 5 mM Tris and 5 mM magnesium sulfate with 80 mM sodium chloride (pH 8.0), while monitoring the absorbance of the eluate at 280 nm. After the baseline was regained, buffer with 135 mM sodium chloride was used to elute the retained PLAP. The enzymatic activity of the eluate was monitored qualitatively during elution and the fractions showing AP activity were pooled (29.5 ml total). The entire volume was dialyzed (MWCO 60 000) twice against 1 l of buffer (5 mM Tris with 5 mM magnesium sulfate, pH 8.0) at 4°C.

2.2.3. Affinity chromatography

For affinity chromatography a plastic column (10 cm×2 cm I.D.) was slurry packed with MIMETIC Blue AP A6XL phase (Affinity Chromatography, Cambridge, UK), which contains a phosphonic group (affinity ligand) bonded onto 6% cross-linked agarose. The Econo equipment (see above) was used for pumping and UV monitoring of the eluate. After equilibration with run buffer (10 mM Tricine, pH 8.5) the sample obtained after IEC (29.5 ml) was applied in two portions allowing PLAP to selectively bind to the affinity material, while unbound proteins were washed from the column using run buffer. The eluate was checked qualitatively for enzymatic activity to verify that no PLAP was eluting. When the UV absorbance was back at the baseline level, the mobile phase was changed to elution buffer (10 mM Tricine with 5 mM potassium phosphate, pH 8.5) and fractions were collected. The collected fractions showing enzymatic activity were pooled giving a total volume of 37.6 ml. After elution the column was washed with 1 M sodium hydroxide for 30 min at 1 ml/min to clean the column.

2.2.4. Centrifugal membrane concentration

The protein concentration of the sample purified by affinity chromatography was increased by centrifugal membrane concentration using Centrisart-C 30 centrifuge filters (Sartorius, Göttingen, Germany) with a MWCO of 10 000. In steps of ca. 4 ml the 37.6-ml sample was concentrated to a final volume of 1.8 ml. The natant showed no enzymatic activity indicating that no PLAP had been lost.

2.3. Analytical techniques

2.3.1. Protein content assay

The total protein concentration of samples was determined according to the method of Lowry et al. [23]. BSA was used for calibration in concentrations of 0–50 µg/ml. Solution D was prepared by mixing 9.6 ml of solution A (8 mg/ml sodium hydroxide and 40 mg/ml sodium carbonate in water) with 0.2 ml of solution B (10 mg/ml copper sulfate in water) and 0.2 ml solution of C (20 mg/ml sodium potassium tartrate in water). To 500 µl of sample, 500 µl of solution D was added, after 10 min followed by 100 µl of Folin reagent (diluted 1:1 with water). Subsequently, the absorbance at 700 nm of the samples was measured in 1.00-cm path-length cuvettes using a Hitachi U-2001 Spectrophotometer (Tokyo, Japan).

2.3.2. Enzymatic activity assay

Activity of the PLAP samples was determined using the enzymatic conversion of pNPP and measuring the absorbance of the resulting yellow product at 405 nm. For the assay 190 µl of a mixture of 0.05 M ammediol (pH 9.8)–100 mM MgCl$_2$

(97.9:2.1, v/v) and 10 μl of 10 mg/ml pNPP were mixed with 5 μl of sample. After incubation for 30 min at 37°C, 1000 μl of 0.1 M NaOH was added to stop the conversion and the absorbance of the samples was measured. The enzymatic activity was expressed in units (U): 1 U corresponds to the conversion of 1 μmol of substrate per min at 37°C. In these calculations the molar absorption coefficient of the product (4-nitrophenol) at 405 nm was taken 18 500 l/mol cm.

2.3.3. Capillary zone electrophoresis

Capillary zone electrophoresis (CZE) experiments were performed on a Beckman P/ACE system 5500 equipped with a diode array detector (Fullerton, CA, USA), using an uncoated fused-silica capillary (Supelco, Bellefonte, PA, USA) of 37 cm (effective length 30 cm)×75 μm I.D. Before use the capillary was, respectively, flushed with 0.1 M NaOH, water and run buffer. The run buffer was 50 mM boric acid with 2 mM putrescine adjusted to pH 8.5 using 1 M sodium hydroxide. The run buffer was filtered through a 0.45-μm membrane filter from Schleicher and Schuell (Dassel, Germany). Between injections the capillary was rinsed with water (1 min) and run buffer (2 min). The separation voltage was 15 kV and the capillary was thermostated at 20°C. Sample was hydrodynamically injected at 0.5 p.s.i. for 5 s, and the data were interpreted at 200 nm using P/ACE Station software (1 p.s.i.=6894.76 Pa).

2.3.4. Capillary gel electrophoresis

CGE experiments were performed at −15 kV on a Bio-Rad Biofocus CE system equipped with a diode array detector using a CE-SDS protein kit (Bio-Rad) including a sieving buffer, a sample buffer and a capillary of 24 cm (effective length 19 cm)×50 μm I.D. Commonly, prior to analysis, 10 μl of sample was mixed 1:1 with sample buffer, and 1 μl benzoic acid (1 mg/ml) and 0.5 μl β-mercaptoethanol was added. The mixture was vortex-mixed and then boiled for 10 min to denature the protein. After cooling, the sample was centrifuged for 2 min at 13 000 rpm, and subsequently introduced into the capillary by electrokinetic injection (−10 kV for 5 s). The following proteins were used for calibration: lysozyme (M_r 14 400), trypsin inhibitor (M_r 21 500), carbonic anhydrase (M_r 31 000), ovalbumin (M_r 45 000), serum albumin (M_r 66 200), phosphorylase (M_r 97 000), β-galactosidase (M_r 116 000) and myosin (M_r 200 000). The calibration lines were constructed by plotting the migration of the calibration proteins relative to benzoic acid against log M_r.

2.3.5. MALDI-TOF-MS

A Micromass VG TofSpec E (Manchester, UK) controlled by Maldi-Tof Version 3.0 software was used to acquire MS data of proteins. The samples were diluted 1:1 in water–acetonitrile (70:30, v/v) with 0.1% TFA. As matrix sinapinic acid (10 mg/ml) was used. For the PLAP of a commercial source a matrix of ferulic acid (20 mg/ml) in 2-propanol–water (1:1, v/v) was also tested.

3. Results and discussion

3.1. Choice of analytical systems

Efficient RP-HPLC of peptides and proteins often requires the addition of TFA to the mobile phase. Since AP is highly unstable under acidic conditions, the use of a TFA-containing eluent for the RP-HPLC analysis of PLAP is not desirable. Therefore, we briefly tested RP-HPLC using a C_4 column (Alltech Macrosphere RP 300, 5 μm) in combination with a neutral phosphate buffer–methanol gradient. However, an increasing backpressure was observed after repeated sample injections, indicating irreversible adsorption of protein molecules to the C_4 stationary phase. Besides, the available sample amounts were rather limited and did not allow repeated injections of 20–50 μl.

CZE can be particularly useful for the separation of closely related protein species and requires small volumes but relatively high concentrations, and we turned to this technique for the analysis of our samples. As starting point a run buffer composed of borate and the modifier 1,4-diaminobutane (putrescine) was taken. Other diamino compounds have been investigated by others [18,24], but putrescine has shown to be particularly useful for the glycoprotein ovalbumin [15,16]. At alkaline pH, PLAP (isoelectric point, pI≈4.5 [2]) will be negatively charged and migrate slower than the electroosmotic

flow. The CZE method was briefly optimized with regard to peak shape by varying the borate concentration (25–100 mM), pH (7.5–9.0) and putrescine concentration (1–4 mM) and injecting the sample collected after the IEC (see next section). Putrescine improves the peak shape and resolution of the observed peaks, but also reduces the electroosmotic flow. A run buffer of 50 mM borate with putrescine at a concentration of 2 mM (adjusted to pH 8.5) gave a satisfactory resolution within a reasonable analysis time (less than 30 min). When no putrescine was added only one rather broad band was observed.

In order to obtain indicative mass information about species present in PLAP samples, CGE was used. It should be noted that the masses as determined by CGE are not fully reliable for glycoproteins. A considerable portion of the glycoprotein mass originates from carbohydrate residues, which do not bind the negatively charged sodium dodecyl sulfate (SDS) molecules [25]. Consequently, the migration time of a glycoprotein will relatively increase so that its molecular mass is overestimated when normal proteins are used for calibration. To achieve more accurate molecular mass determination of PLAP, MALDI-TOF-MS analysis was carried out, also enabling the identification of other entities present in the isolated samples. To check for total protein content of the retrieved sample the method of Lowry et al. was used [23]. For the estimation of the enzymatic activity of samples an assay based on the conversion of 4-nitrophenyl phosphate into a yellow product was applied.

3.2. Analysis of PLAP after ion-exchange liquid chromatographic purification

In first instance, PLAP was isolated from a crude human placenta extract using IEC, which has previously been used for purification of alkaline phosphatase from various sources [26–31]. After loading the crude sample on the cellulose based DEAE-52 column, PLAP was eluted by applying a salt step-gradient. Fifteen fractions (2 ml each) collected from the column showed enzymatic activity and were pooled and dialyzed. With a Lowry assay, the protein content was determined to be 0.84 mg/ml. The enzymatic activity of the pooled fractions appeared

to be 5.7 U/ml, giving a specific activity of 6.8 U/mg protein. This is considerably lower than for PLAP of commercial sources, which claim a specific activity of approx. 15 U/mg. Upon CZE analysis of the collected sample, several (partly overlapping) peaks were found (Fig. 1A). This might indicate that the sample probably also contains several other proteins. The first two peaks in the electropherogram originate from the buffer used to dialyze the DEAE fractions.

In order to gain information on the molecular mass of the sample constituents, CGE and MALDI-TOF-MS were also employed. CGE analysis (Fig. 2A) revealed two protein peaks at 8.1 and 8.7 min, respectively. The molecular mass of the first peak was estimated to be M_r 69 000, which is close to the
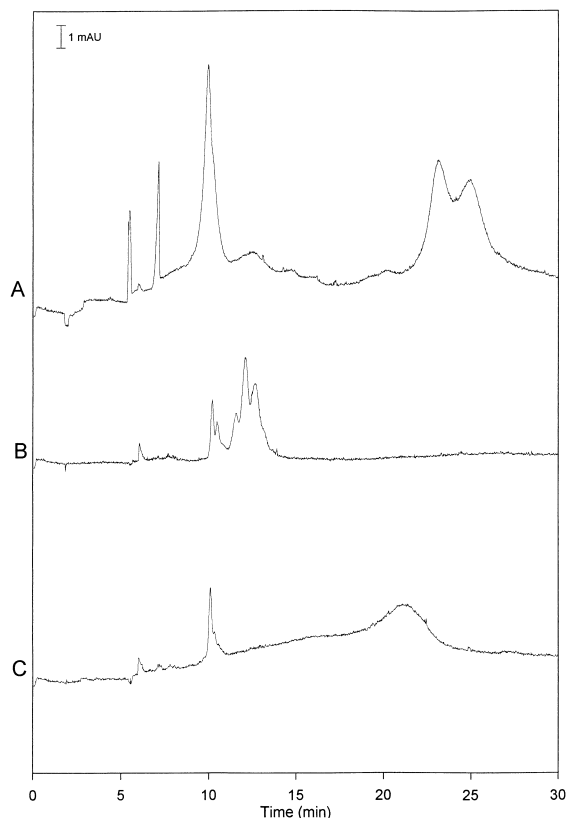

Fig. 1. CZE of PLAP samples. (A) Sample obtained after IEC of placental extracts; (B) sample obtained after successive IEC, AC and CMC of placental extracts; (C) commercially available PLAP sample. Conditions, see Experimental section.
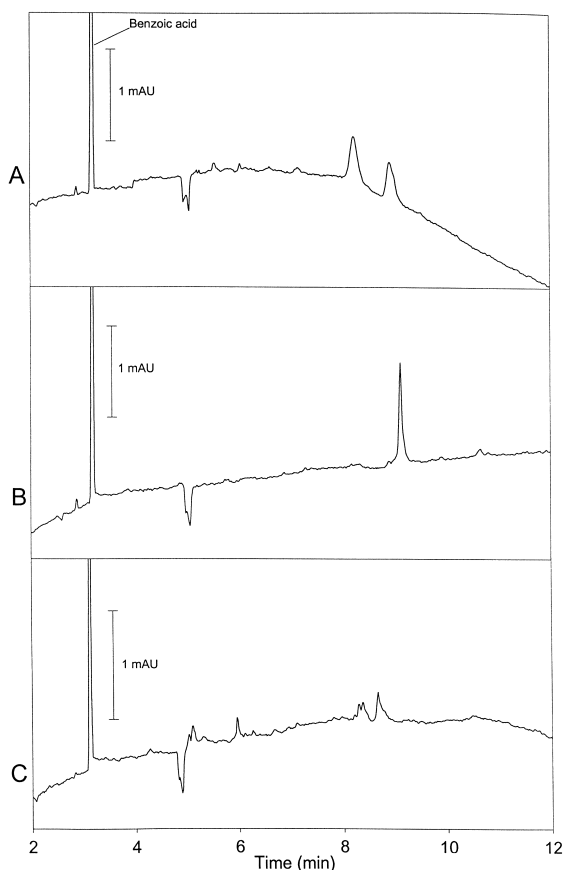
Fig. 2. CGE of PLAP samples. (A) Sample obtained after IEC of placental extracts; (B) sample obtained after successive IEC, AC and CMC of placental extracts; (C) commercially available PLAP sample. Conditions, see Experimental section.
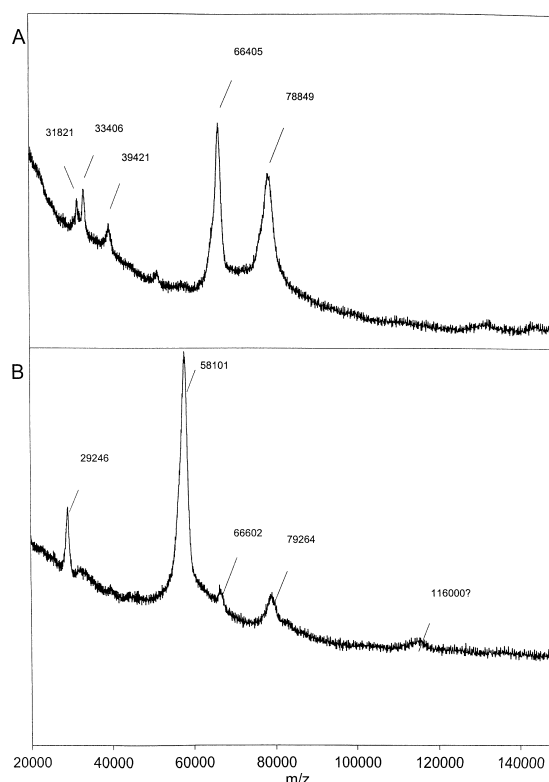

Fig. 3. MALDI-TOF-MS of PLAP samples. (A) Sample obtained after IEC of placental extracts; (B) sample obtained after successive IEC, AC and CMC of placental extracts. The given m/z values are the centroids as determined on 80% peak height; conditions, see Experimental section.

molecular mass of human serum albumin (HSA), i.e., M_r 66 000. Upon addition of pure HSA to the sample and subsequent analysis the first peak increased and no extra peaks were observed. The molecular mass of the second peak was estimated to be M_r 95 000. Analysis of the same sample with MALDI-TOF-MS (Fig. 3A) revealed the presence of two major constituents with molecular masses of 66 405 and 78 849, respectively (based on centroid measurements). This observation also indicates that HSA (M_r 66 000) might be one of the major sample components. The identity of the other component (M_r 79 000) was not established, but it might correspond to the second peak found with CGE. The masses

found using CGE and MALDI-TOF-MS differ substantially for the larger component (M_r 95 000 and 79 000, respectively). A plausible explanation for this is that CGE can give erroneous estimations of the molecular masses of glycoproteins due to the presence of sugar entities, as was outlined in the previous section [26]. Surprisingly, no PLAP was indicated by MALDI-TOF since no peak was observed at approximately m/z 60 000 (i.e., the molecular mass of a PLAP subunit [1,2]). Using the stated MALDI sample preparation dissociation of PLAP into its subunits is very likely. This result suggests that the DEAE eluate contains relatively little PLAP and mainly other proteins, although suppression effects causing a low MS response cannot be ruled out.

3.3. Analysis of PLAP after affinity chromatographic purification and centrifugal membrane concentration

In order to further purify the PLAP sample, the pooled DEAE and dialyzed fractions were subjected to AP affinity chromatography [32,33] on a new MIMETIC Blue AP phase. A total of 29.5 ml was applied in two portions of ca. 15 ml onto the affinity column. After each portion the column was eluted with phosphate-containing buffer and a total volume of 37.6 ml was collected. Subsequent Lowry analysis indicated that the protein content was only 25 μg/ ml, while the enzymatic assay revealed an activity of 4.1 U/ml. If dilution (29.5 ml applied vs. 37.6 ml eluted) is taken into account, the PLAP recovery in terms of activity is calculated to be 92%, which means that the loss of PLAP in this procedure is very small. In contrast, most of the proteins (97%) were removed by the AP affinity column. In fact, assuming that the sample obtained after affinity chromatography does not contain other proteins than PLAP, the Lowry assay shows that only 3% of the protein present in the DEAE-52 fractions is PLAP, which explains the absence of detectable PLAP signals during CGE and MALDI-TOF analysis. The final PLAP concentration of the purified sample actually is rather low and, therefore, it was necessary to increase the protein concentration to allow further analysis by CZE, CGE and MS. For this purpose, a centrifugal membrane concentrator was used with a MWCO of 10 000. With this device, the sample volume was decreased from 37.6 to 1.8 ml while retaining all proteins. This was confirmed by Lowry analysis of the concentrated sample, which indicated a protein concentration increase of a factor 21 (from 25 μg/ml to 0.53 mg/ml), which perfectly matches the achieved concentration factor. The gain in enzymatic activity (from 4.1 to 68 U/ml) was somewhat less (factor 16.6) but still considerable, resulting in a specific activity of 128 U/mg. Apparently, the PLAP activity is not fully recovered in the applied concentration procedure. Fig. 4A and B summarize the PLAP purification and concentration procedures in terms of protein concentration and enzymatic activity of the intermediate fractions.

In order to check the purity of the concentrated sample, MALDI-TOF-MS, CGE and CZE analysis
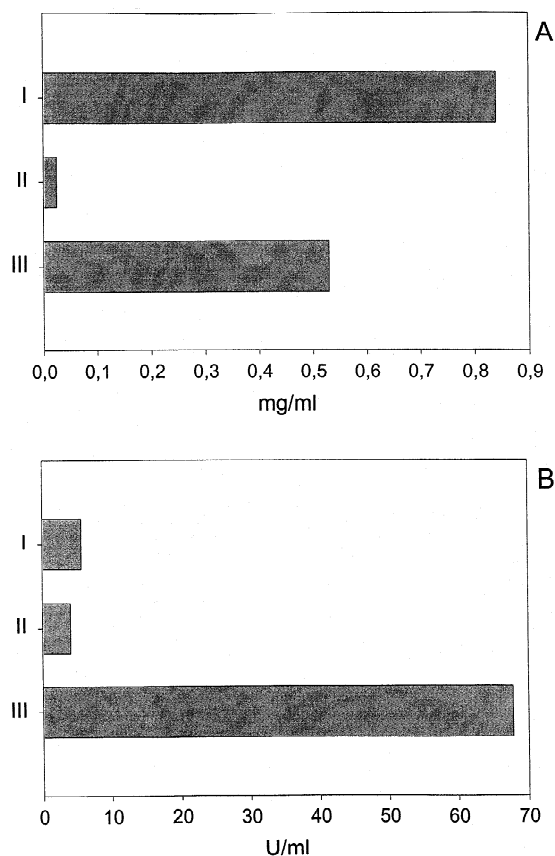

Fig. 4. Protein concentration (A) and enzymatic activity (B) of PLAP samples obtained from placental extracts. (I) Sample obtained after IEC; (II) sample obtained after successive IEC and AC; (III) sample obtained after successive IEC, AC and CMC. Conditions, see Experimental section.

were carried out. The major peaks found in the MALDI-TOF mass spectrum at m/z 58 101 and 29 246 (centroid values) (Fig. 3B) indicate that the purification procedure indeed had been successful. These peaks are most likely due to the singly and doubly charged subunits of PLAP. Averaging the centroid values reveals a molecular mass of the PLAP subunit of M_r 58 294 Da. In addition, two minor peaks were detected at m/z 66 602 and 79 264, respectively. These reveal that the two major components of the DEAE fractions (HSA and an unknown protein) are still present in the purified sample, but relatively in much lower quantities than before. The relative purity of the concentrated PLAP

sample is also supported by CGE analysis, which shows only one peak when the sample is denatured (i.e., boiled after addition of β-mercaptoethanol) prior to analysis (Fig. 2B). The mass indicated by the CGE calibration (M_r 96 000) does not match the molecular mass of PLAP, but this might be caused by the fact that PLAP is a glycoprotein (see above). The purity of the PLAP sample was also checked by CZE (Fig. 1B). Indeed, a part of the major peaks observed in the DEAE fraction (Fig. 1A) were not present now. But, remarkably, not one peak but several closely spaced peaks showed up. Probably, CZE reveals the microheterogeneity of PLAP, i.e., the cluster of peaks might be caused by several glycoforms and/or oligomers of the enzyme.

3.4. Analysis of PLAP from a commercial source

From the experiments described above, it can be concluded that with a procedure of subsequent IEC, affinity chromatography and centrifugal membrane concentration, highly pure PLAP can be recovered from human placenta. However, since this procedure is quite laborious, we considered the use of PLAP obtained from a commercial source. To check the purity of this sample and to characterize it, we applied the same set of techniques as used to monitor the isolation procedure. A 1.00-mg/ml solution of PLAP sample was subjected to Lowry and activity analysis. The protein content was found to be 0.77 mg/ml, while the enzymatic activity of the same sample was 11.9 U/ml, so that the specific activity was calculated to be 15.6 U/mg, which is in line with the activity claimed by the manufacturer (ca. 15 U/mg). Compared to the PLAP purified in our laboratory (128 U/mg) this specific activity is considerably lower, but still higher than the value obtained for the PLAP sample obtained by only applying IEC (6.8 U/mg). These results indicate that the PLAP from commercial source might contain considerable amounts of other proteins.

The sample was also analyzed by CGE after first treating it with mercaptoethanol at high temperature. The electropherogram showed five peaks corresponding to estimated molecular masses of 13 000, 22 000, 75 000, 78 000 and 91 000 (Fig. 2C). In fact, the peak corresponding to M_r 91 000 is close to the M_r 96 000 found for the purified and concentrated

PLAP and just within the experimental variation given by the repeatability of the method (RSD=6%, data not shown). The presence of various peaks suggests that the commercial PLAP contains several other proteins, and explains why its specific activity is rather low. Unfortunately, we were not successful in our attempts to get good MALDI-TOF-MS spectra from the commercial PLAP sample. Despite several attempts using two different matrices (sinapinic acid and ferulic acid) in several ratios to the sample, no signal corresponding to the m/z of a PLAP subunit (M_r 58 000) was found. In fact, it was not possible to produce a reliable spectrum of this sample, which might be caused by the fact that the sample contained too much salt.

The commercial PLAP sample was also analyzed by CZE (Fig. 1C) revealing only one peak in the 9–13 min region and not a cluster of peaks as found for the purified sample (cf. Fig. 1B). This observation might indicate that the larger part of the PLAP present in the commercial sample is comprised of one glycoform only. This conclusion is supported by the fact that a similar correlation between the peak area (corrected for migration time) of the PLAP peaks and the measured activity is found for both the purified and the commercial PLAP sample (Fig. 1B and C). Based on the above mentioned values for activity of the purified PLAP sample (68 U/ml) and the commercial PLAP sample (11.9 U/ml), and assuming a constant injection volume, a peak area–activity ratio of 59 (arbitrary units) was determined for the purified sample, while for the commercial sample a ratio of 51 was calculated. In other words, the band at 10 min in Fig. 1C indeed seems to originate from PLAP.

4. Conclusions

The combination of several analytical techniques, i.e., enzymatic activity and protein concentration assay, CZE, CGE and MALDI-TOF-MS, is a powerful approach to investigate the purity and composition of enzyme samples. Applying this combination it was shown that IEC alone is surely not sufficient to isolate alkaline phosphatase from human placenta, but that an additional purification by affinity chromatography is required. In addition, it was

demonstrated that also commercial PLAP samples may contain several other proteins next to alkaline phosphatase. Clearly such observations are essential when the use of PLAP for medical purposes is studied and PLAP samples are used for in vivo experiments. In such situations characterization of samples by enzymatic activity assays only is fully inadequate. This study also indicates the potential of CGE, and particularly CZE to reveal the micro-heterogeneity and macroheterogeneity (e.g., glyco-forms, oligomers) of proteins. Currently, we are investigating CZE as a means of monitoring the (forced) degradation of AP.

Acknowledgements

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