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Purification and characterization of Alpha-Fetoprotein from the human hepatoblastoma HepG2 cell line in serum-free medium

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Abstract Alpha-fetoprotein (AFP) is a tumor-associated embryonic molecule whose precise biological function remains unclear. A complete definition of the physiological activities of this oncofetal protein has been severely limited, until now, by the lack of a purification procedure appropriate to obtain pure AFP in appreciable amount. The present report describes a purification procedure extremely rapid and simple and takes advantage of the well-known fact that AFP contains copper. We have developed a single-step purification procedure by immobilized copper-chelate affinity chromatography using the culture medium from human hepatoblastoma cell line HepG2 grown in the absence of serum. This method yields AFP at high purity and high yield. Purified AFP amino acid sequence, molecular mass, carbohydrate structure, and copper content were found to be in line with previous studies. Moreover, we found that the purified AFP has superoxide dismutase activity with efficiency similar to that of the native Cu, Zn SODs at physiological pH. This result may provide further support to the idea that AFP is a bifunctional protein, acting in cellular defence against oxidative stress both as a copper buffer and as a superoxide radical scavenger.

Keywords Serum-free medium · Human hepatoblastoma · Alpha-fetoprotein · Copper · Superoxide dismutase

Abbreviations

AFP	alpha-fetoprotein
Cu Zn SOD	Cu, Zn superoxide dismutase

Introduction

Oncofetal antigens represent a special category among the tumor-associated antigens, because they occur in the fetus but are absent or present only in low concentration in healthy adult individuals. These antigens are of interest for developing diagnostic methods and immunotherapy for several kinds of tumor. Alpha-fetoprotein (AFP) is the major serum protein synthesized during fetal life and its content decreases rapidly during early postnatal life (Ruoslahti and Terry, 1976). Since its relationship to cancer was reported in the mid-1960s, this tumor-associated fetal protein has demonstrated clinical utility both as a tumor

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marker and a birth defect screening agent (Deutsch, 1991). Although the physicochemical and structural properties of this 70-kDa glycoprotein have been extensively described, only in vitro functional roles of this oncofetal protein have been ascertained to date. Such physiological properties of the oncofetal protein have encompassed mainly ligand carrier/transport functions and modulation of in vitro immune response assays (Ogata et al., 1995; Deutsch et al., 2000). Indeed, AFP has been shown to bind in vitro to various substances, some of which serve as ligands for members of the steroid/thyroid nuclear receptor superfamily. However, other ligands binding to AFP (rodent and human) include bilirubin, metabolic dyes, L-tryptophan, wafarin, triazine dyes, phenylbutazone, streptomycin, phenytoin, anilinonaphthaline sulphate, heavy metals, alcohols, and drugs (Moskaleva et al., 1997; Deutsch et al., 2000; Mizejewski, 2001). Owing to this multiplicity of carrier/transport reports, some investigators still hold that AFP is merely the fetal counterpart of serum albumin, as was previously proposed (Ruoslahti and Terry, 1976).

In the last decade, the growth regulatory properties of AFP have aroused interest as a result of studies involving ontogenetic and oncogenic growth in both cell cultures and animal models. A myriad of studies have now documented that AFP is capable of regulating growth in ovarian, placental, uterine, hepatic, phagocytic, bone marrow, and lymphatic cells (Toder et al., 1983; Mizejewski et al., 1990) in addition to various neoplastic cells (i.e., MCF-7 and MTW9A breast cancer; Bennett et al., 1998; Dudich et al., 1998). In the authors view, AFP should no longer be considered merely a fetal form of albumin only to be employed as a marker for cancer and fetal disorders; rather, AFP should now be considered as a possible direct or indirect factor associated with the regulation of growth, differentiation, regeneration, and transformation in both ontogenetic and oncogenic growth processes.

Recently, mice lacking AFP have been generated to assess its developmental role and physiological function in vivo. It has been reported that AFP is not required for development but plays a critical and non-redundant role in the female reproductive system (Gabant et al., 2002). However, the biological role of this major embryonic serum protein is still unknown although numerous speculations have been made.

A more complete understanding of these and other AFP-mediated effects, including its influence on cell differentiation, could be considerably advanced by the definition of the structural features of the AFP molecule that are responsible for these biological activities. However, implementation of these studies has been made difficult by some problems. Separation of AFP from serum albumin, which is generally present in the starting materials used for purification, is difficult since there is a great similarity in molecular and structural properties of the two proteins. Therefore, most procedures include a specific immunoaffinity step, at some stage, to achieve complete purification (Murgita and Wigzell, 1981; Dudich et al., 1999). Although yields have been improved by the use of low-affinity antisera preparations, it is still difficult to establish conditions under which the recovery and the original conformation of the protein are not affected by the elution buffers (Uversky et al., 1995; Tomashevski et al., 1998; Mizejewski, 1997). Furthermore, the yield of material remains relatively small, due to the number of steps, which are generally necessary. In addition, the variability in the starting materials for purification, i.e., serum or ascitic fluid from patients with hepatocarcinoma and fetal or cord serum, could also result in protein heterogeneity in its carbohydrate content, extent of degradation, and association with prostaglandins or fatty acids (Parmelee et al., 1978; Ferranti et al., 1995). These modifications of the protein may be related to different post-translation processing occurring in the tumor or organ of origin or to the duration of its presence in the different fluids in which the protein is normally found (Pucci et al., 1991).

Recently, recombinant AFP (rAFP) has been expressed in *Escherichia coli* and purified by a fractionation procedure allowing eliminating the contaminants but, more importantly, to separate aggregated populations of rAFP from monomer rAFP formed by inclusion bodies (Boismenu

et al., 1997). The molecular mass determined by electrospray mass spectrometry (ES-MS) was approximately 4% less than the 68,803 Da determined for native human AFP derived from a hepatoblastoma cell line (Ferranti et al., 1995). This difference was ascribed to the absence in the *E. coli* rAFP of the carbohydrate structures that are present on mammalian-derived AFP; thus, rAFP is not helpful for enzyme immunoassay used to detect many kinds of disease biomarkers in medical laboratories and hospitals (Tamano et al., 2005). However, the whole purification usually requires a few days to be completed and the recovery of purified monomer rAFP was not particularly high (0.5–1 mg/l of bacterial culture).

At present, AFP for laboratory investigations is mainly prepared from early stage abortuses and from umbilical cord blood serum collections. The protein also appears in relatively large amounts in the serum of patients with primary hepatocellular carcinomas and some gonad tumors. These sources do not appear able to be a reliable source of the amounts of protein that are required in clinical applications and the purified protein is very expensive (Deutsch et al., 2000).

Recently, a number of laboratories found serum-free medium of HepG2 cells as a potential source of human AFP (Tecce and Terrana, 1988; Ferranti et al., 1995; Deutsch et al., 2000). However, the methods used in these studies to purify AFP are not easily reproducible; therefore, it is still worth to develop purification methods to isolate AFP. We have tried to overcome this obstacle by using a rapid and gentle method of purification of AFP from serum-free medium of human HepG2 cell line. Our method has isolated a high amount of AFP (15 mg/l of culture medium) to 95% purity and it does not alter structural or functional parameters of the protein that were very similar to those of AFP from other natural sources. Moreover, the biochemical, spectroscopic and catalytic properties of the purified protein indicate that AFP has properties similar to that of Cu, Zn SOD at physiological pH, leading to address AFP as a bi-functional protein: copper carrier and antioxidant enzyme.

Materials and methods

Cell culture

Human hepatoblastoma HepG2 cells were purchased from the American Type Culture Collection (Manassas, Virginia, USA) and grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin and streptomycin and at 37 °C in an atmosphere of 5% CO_2 in air. Cells were routinely trypsinized and viability was assessed by Trypan blue exclusion. For purification of AFP, after 24 h plaiting, cells medium was discarded and cells washed with PBS and grown in a serum-free medium. The culture supernatant was collected at 72 h of growth. Detached cells were removed from the medium by centrifugation (1,000 *g* for 20 min at 4 °C). After 72 h from serum withdrawal no significant changes in growth and viability of HepG2 cells were observed with respect to cells grown in the presence of serum, as stated direct cell counts using Trypan blue exclusion.

Purification of AFP from serum-free medium of HepG2 cell line

AFP was purified from culture supernatant of HepG2 cells with a single-step passage on metal-chelate chromatography (IMAC, Pharmacia) in a gravity-fed column saturated with a 20 mM $CuSO_4$. The serum-free medium (1,500 ml) was concentrated with a Minitan Ultra filtration System (Millipore, Molsheim, France), with a tangential flow device on an ultra filtration membrane (cut-off of 10,000 Da). The concentrated sample was dialyzed against binding buffer (20 mM Na_2HPO_4, 1 M NaCl, pH 7.2). After dialysis the sample was cromatographed on an IMAC resin in a gravity-fed column, previously equilibrated with the same buffer. Adsorbed AFP was eluted with elution buffer (20 mM $NaHPO_4$ pH 7.2, 1 M NH_4Cl).

Fractions containing AFP were concentrated by exhaustive dialysis against bidistilled water, previously incubated overnight with Chelex 100 resin (5 g/100 ml) (Bio-Rad, Hercules, CA, USA) to remove traces of copper.

The sample was then chromatographed by gel filtration on a Superose 12 HR 10/30 column connected to an FPLC system (Pharmacia) at 0.3 ml/min.

Purity of AFP was assessed by polyacrylamide gel electrophoresis (SDS–PAGE) in a slab gel system from Bio-Rad. SDS–PAGE was performed according to Laemli (Laemmli, 1970) on 12% acrylamide gels. The samples were heated at 100 °C for 5 min in presence of 2% SDS and 5% 2-mercaptoethanol before applying to the gel. Molecular weight of AFP was identified by comparison with molecular weight standards (Bio-Rad), run under the same conditions. For immunological determination, the gels were blotted on a nitrocellulose membrane (0.45 μm, Bio-Rad Labs., Hercules, CA, USA) using the Bio-Rad Transblot apparatus. AFP was detected by using a 1:500 of the monoclonal anti-human AFP (goat) (Calbiochem).

The immune reaction was visualized with the Immuno-Blot Horseradish Peroxidase Assay Kit (Bio-Rad, Labs, Hercules, CA, USA). The isoelectric points were determined by isoelectric focusing on 4% polyacrylamide gels, containing 3% ampholines (LKB), pH range 3.5–9.5 (Righetti and Drysdale, 1971). Bands of protein were stained with Coomassie blue R-250 and bands of SOD activity were revealed according to Beauchamp and Fridovich (1971).

Double diffusion was used for qualitative evaluation of AFP and human Cu, Zn SOD using anti-AFP antibody reaction according to the Ouchterlony technique (Ouchterlony, 1967). The AFP and human Cu, Zn SOD were placed in upper wells and anti AFP solution in the lower wells punched in agarose gel 1% on the glass plate using Multiphor II electrophoresis unit. After, the plate was placed in humidity chamber and it was leaved at room temperature for 24 h. The precipitation lines were visualized with Coomassie R-250.

Primary structures analysis of AFP after purification

Electro spray mass spectrometric (ESI MS) analysis for the intact protein was performed with a Q-TOF hybrid mass spectrometer (Waters, the Netherlands). Protein and peptide samples (10 ml, 2.5–5.0 pmol) were first desalted by a Zip Tip C18 (Millipore) cartridge and then injected into the electro spray nebulizer at a flow rate of 2 μl/min. The spectra were obtained by scanning from 2,000 u to 400 u at 2 s/scan. Mass scale calibration was carried out by using the multiple charged ions of a separate injection of myoglobin. The amount of the various components was assessed by integration of the multiple charged ions of the single molecular species. The signals recorded in the spectra were associated to the corresponding peptides on the basis of the expected molecular weights by using Bio Lynx software provided by the instrument manufacturer.

Trypsin hydrolysis

AFP was reduced and alkalized as reported in Ferranti et al. (1995). Trypsin hydrolysis was carried out in 0.4% ammonium bicarbonate, pH 8.5, at 37 °C for 4 h using TPCK-trypsin (Sigma) with an enzyme/protein ratio of 1:50. At the end of the incubation time the reaction mixtures were immediately freeze-dried.

MALDI MS analysis

Alpha-cyano-4-hydroxy cinnamic acid (Fluka, CH-9471 Buchs SG1, Switzerland) was used as matrix; the protein or peptide samples (1 μl from a solution 1 g/l in water) were loaded on the target and dried. Afterwards, 1 μl of a solution containing 10 mg/ml of matrix in a mixture of water/acetonitrile/TFA (50/50/0.1) was added to the sample. The samples were analyzed with a Voyager DE-Pro mass spectrometer (PerSeptive Bio system, Framingham, MA, 01701, USA) operating either in linear or in reflector mode.

Analysis of spectroscopic parameters and copper content

Optical measurements were performed on Perkin–Elmer Lambda 9 spectrometer and the ultraviolet extinction coefficient at 280 nm employed for 1% solution of AFP was 5.4. This coefficient was based on the determination of highly purified

samples standardized by micro-biuret and it is in good agreement with the extinction obtained by Ruoslahti et al. (1974).

ESR spectra were recorded with a Bruker ESP 300 spectrometer. Paramagnetic copper content was calculated with Cu-EDTA as standard. Copper was measured by atomic absorption spectrophotometer using a Perkin–Elmer 2100 atomic absorption apparatus, equipped with a graphite furnace with platform. Before analysis the samples were digested by treatment with an equal volume of 65% nitric acid at room temperature for a least 1 week.

Determination of superoxide dismutase activity

Activity assays were carried out either by a spectrophotometric procedure, following the inhibition of cytochrome c reduction by superoxide dismutase (in Phosphate Buffer 50 mM, pH 7.8, $\mu = 0.13$ and Hepes buffer, 50 mM, pH 7.4, $\mu = 0.03$ or 0.013) (Beyer et al., 1986), or by a polarographic method with an AMEL polarographic analyzer (Model 466), that directly measures the catalytic dismutation rate constant following the superoxide-dependent increase of the limiting current, due to O_2 reduction to a dropping mercury electrode (in Tetraborate buffer 100 mM, pH 9.8) (Rigo et al., 1975). The SOD activity was expressed as function of the copper content, the metal responsible for the catalytic function of the enzyme that not always is present at a ratio of 0.5 protein/copper (Steinkuhler et al., 1991). The polarographic method was also used to measure the kinetic constant of O_2^- dismutation of AFP.

Proteins were determined by a micro Biuret method (Goa, 1953). Statistical analysis of data was performed by the Student's *t*-test; *P* values <0.05 were considered significant.

Results and discussion

The focus of the investigation was to develop a method for the isolation of AFP from serum-free medium of HepG2 cells that retains the structural and functional integrity of its native protein counterpart. We have used the HepG2 cells, both for their capacity to secrete the AFP in the medium and to grow in the absence of serum (Tecce and Terrana, 1988). The reason for this was to reduce the level of total contaminating proteins and to avoid production of bovine AFP eventually present in the serum. No significant changes in growth and viability were observed with respect to HepG2 cells grown in the presence of serum as stated by Trypan blue counts (data not shown). We found that the medium collected from these cultures offered the advantage to produce increasing amount of AFP in a time-dependent way (Fig. 1A).

Based on the evidence that AFP contains copper (Aoyagi et al., 1978), we have found a method with a single-step passage on a metal-chelate affinity chromatography for the purification of AFP from serum-free medium of HepG2 cells. Figure 1B shows the elution profile with one well-resolved peak (II pool) and a minor peak (I pool). The rapidity of this gentle procedure allowed us to obtain from II pool a single major component; in fact, as demonstrated by SDS–PAGE, only one major band was observed by overloading the sample. Other two faint bands were detected but they do not account for more than the 5% of the total proteins (Fig. 1C). This band with apparent molecular weight of 68,000 Da corresponds to AFP as demonstrated by Western-blot analysis (Fig. 1D). Purity and native apparent molecular masses were also determined by gel filtration (Fig. 2A) and by electro spray mass spectrometry. The transformed mass spectrum is shown in Fig. 2B: analysis of the spectrum confirmed that the protein was a mixture of at least two species, whose measured mass (68,803 and 68,654, ratio 4/1) perfectly matched with that of the reported sequence of AFP (Morinaga et al., 1983), considering the presence of a N-linked oligosaccharide moiety fucosylated and unfucosylated respectively (Ferranti et al., 1995). To obtain full structural characterization, the protein mixture was reduced, carboxymethylated, and the peptides, after HPLC, were analyzed by MALDI MS. With this single experiment it was possible to verify the entire amino acid sequence (data not shown), which resulted identical to that predicted on the basis of previous

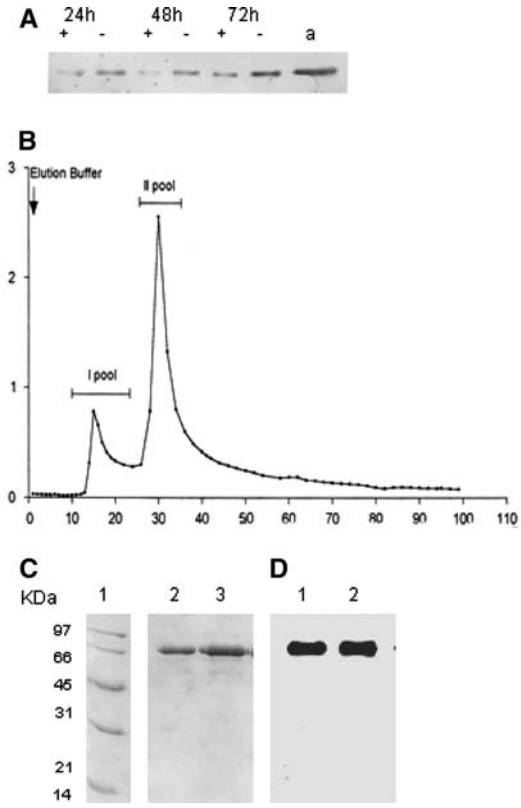

Fig. 1 A) Western-blot analysis of AFP in the culture medium of HepG2 cells at 24, 48, and 72 h (with: + or without: – serum medium; a: purified AFP). Each lane contains 5 μg of total proteins. **B)** Chromatogram obtained from immobilized metal ion affinity (IMAC) of culture supernatant from HepG2 cells at 72 h. The culture supernatant was applied to IMAC column saturated with 20 mM $CuSO_4$ and equilibrated with 20 mM Na_2HPO_4, 1 M NaCl, pH 7.2 (Binding Buffer). The column was washed with 100 ml of binding buffer and proteins were eluted with 20 mM Na_2HPO_4, 1 M NH_4Cl, pH 7.2 (Elution Buffer). Protein absorbance was monitored at 280 nm and collected in two pools (I and II). **C)** SDS–PAGE (12% gel) analysis of AFP after the purification from IMAC. Molecular weight markers (lane 1), II pool of IMAC: 5 μg (lane 2) and 10 μg (lane 3). **D)** Western-blotting analysis with a monoclonal antibody against AFP of the II pool of IMAC: 5 μg (lane 4) and purified AFP (lane 5)

studies (Morinaga et al., 1983; Pucci et al., 1991). Furthermore, the presence of a carbohydrate chain in the purified protein perfectly agreed with the shift of the AFP band of an apparent molecular weight of 3,000 Da lower after oligosaccharide release by PNGase F treatment (Fig. 2C).

We found that purified AFP has one mol copper/mol protein (Aoyagi et al., 1978). Double integration of the copper EPR spectra carried out on purified AFP indicates that the protein has about 98% of the copper expected on a 1 mol copper/mol protein (Fig. 3—spectrum *c*). The EPR spectrum of AFP showed a pattern typical of nearly tetragonal symmetry ($g_{II} > g_{\perp}$) having well resolved parallel hyperfine lines and A_{II} value. These magnetic values are typical of about three-nitrogen legation around Cu(II) as reported for the synthesis N-terminal peptide of human AFP (Lau et al., 1989) and similarly to that of Cu, Zn SODs (Carrì et al., 1994) (Table 1). Due to the peculiar copper coordination we investigate whether AFP could show superoxide dismutase activity. Polarographic determination of purified AFP showed that it is able to efficiently dismutate the superoxide with a catalytic constant of 0.5×10^9 $M^{-1}s^{-1}$, when expressed on the basis of copper content at pH 9.8 (Table 1). However, the value obtained for AFP was significantly lower than that of the bovine Cu, Zn SOD and slightly lower than that of the human Cu, Zn SOD. Moreover, the AFP activity was identical to that of the human recombinant Cu, Zn SOD (Steinkuhler et al., 1994). In order to assess if the dismutating activity was also present at more physiological pH we performed specific activity measurements at different pH (7.4 and 7.8) and ionic strength values (0.03 and 0.13). In particular, we carried out spectofotometric assays both in phosphate and Hepes buffer. Ionic strength was adjusted by means of the addition of sodium bromide, which was also useful to avoid interference of Cl^-. The dismutase-like activity of AFP was also maintained under these conditions; in particular, AFP activity was lower than that of the bovine enzyme but comparable with that of the human Cu, Zn SOD (Table 2). AFP displayed superoxide dismutase activity also using the inhibition of NBT photo-reduction gel staining method (Fig. 4A). A pI (4.8 was determined for the major component of HepG2 AFP with superoxide dismutase activity. A small amount of a more basic component devoid of SOD activity is visible in the Coomassie stained gel, likely attributable to the apo-AFP form. AFP molecular transition forms have been reported at

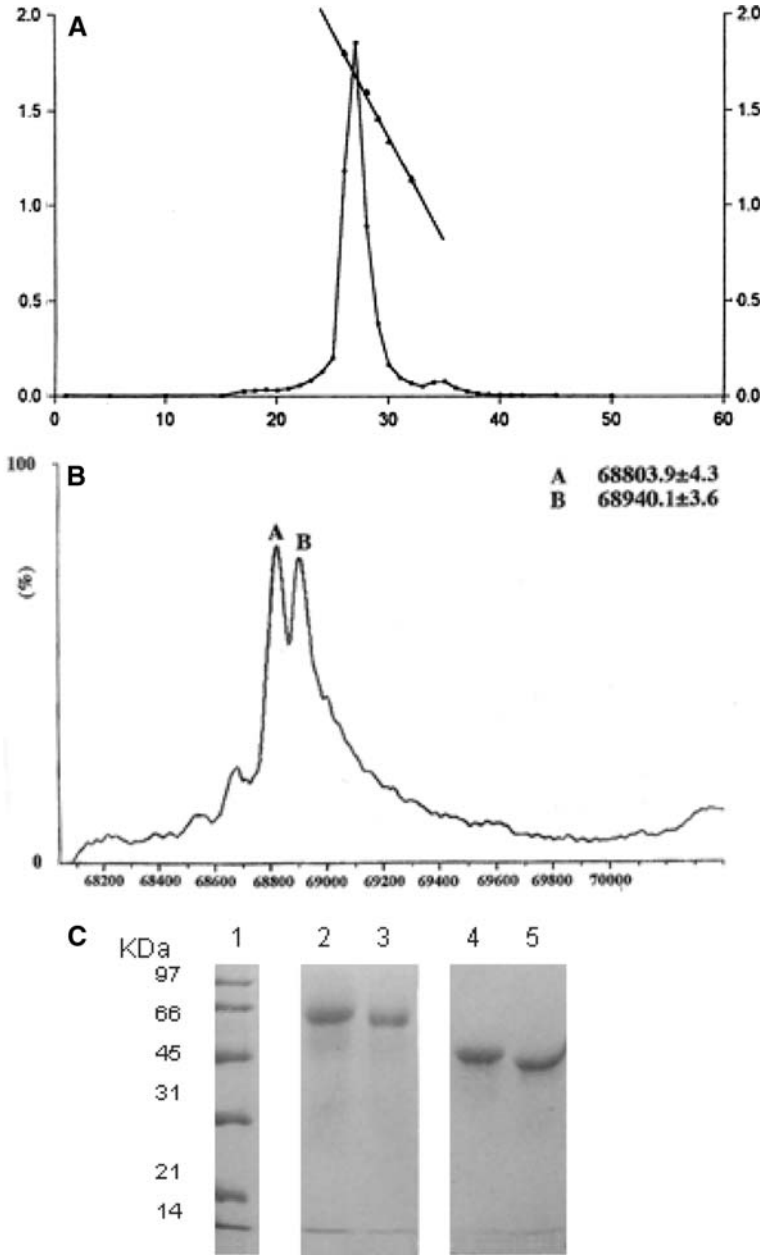

Fig. 2 A) Gel filtration by chromatography on Superose 12 HR 10/30 by FPLC. AFP after IMAC was chromatographed on Superose 12 HR 10/30 column. The proteins were eluted with 50 mM sodium phosphate, 150 mM NaCl at pH 7.2. The column was calibrated with alcohol dehydrogenase of yeast (150,000 Da), albumin (67,000 Da), ovalbumin (43,000 Da), chimotrypsinogen A (25,000 Da), and ribonuclease A (13,000 Da). The void volume was measured by Blue Destran 2,000 Da. **B)** Electrospray mass spectrum of purified AFP. The spectrum is in the trasformed mass scale. Molecular masses are reported as average masses. The two peaks correspond to glycosilated and deglycosilated species of AFP. **C)** Estimation of the number of glycosylation sites on purified AFP. The protein (5 μg) was denatured at 100 °C for 5 min. and after was digested with PNGase F (1.25 mU), incubated overnight at 30 °C. Molecular weight markers (lane 1); untreated AFP (lane 2); PNGase-treated AFP (lane 3); untreated ovalbumin (lane 4) and PNGase-treated ovalbumin (lane 5). Five micrograms of protein was applied to each lane

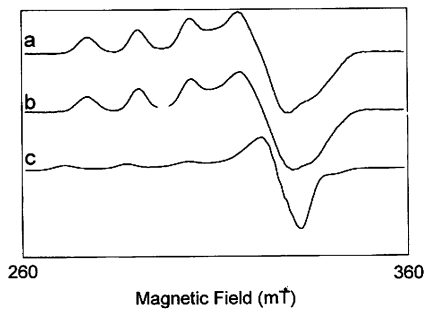

Fig. 3 ESR spectra of purified AFP (c) BSOD (a) and HSOD (b). The proteins were dissolved in 10 mM phosphate buffer, pH 7.4. Protein concentration was 3.3×10^{-4} M. EPR conditions were: microwave power, 20 mW; modulation amplitude, 1 MT; microwave frequency, 9, 43 GHz; temperature, 100 K

both acidic and basic extremes of the pH range and ionic strength (Zizkovsky et al., 1983; Deutsh et al., 2000) providing a likely explanation for the lower value of the catalytic constant of AFP at pH 9.8.

Double immunodiffusion analysis of purified AFP showed a partial identity with the human Cu, Zn SOD, and single spur between two proteins was observed with monoclonal anti-AFP (Fig. 4B). AFP displays a homology with human Cu, Zn SOD on domain 1 (31% identity over 32 amino acids) (Mizejewski, 1997). The cross-reactivity of AFP antibody with Cu, Zn SOD was, however, unusual due to short sequence homology between the two proteins (AFP, region 232–245 aa; Cu, Zn SOD, region 42–52 aa) (Fig. 4C). In this context, we previously demonstrated that the region binding copper (region 42–52 aa) of Cu,Zn SOD is highly antigenic (Ciriolo et al., 1994), therefore it could be sufficient to be recognized by the monoclonal anti-AFP.

In conclusion, in this paper we have reported a single-step purification method that permits to obtain a good amount of AFP from HepG2 cell line with molecular and functional parameters as reported in literature. Moreover, the characteristic properties similar to that of Cu, Zn SOD may provide support to the idea that AFP is a bifunctional protein, acting in cellular defence against oxidative stress both as a copper transporting and as a superoxide radical scavenger.

Table 1 Properties of AFP compared to the bovine and the human Cu, Zn SOD

	Mass (kDa)	pI	Cu/protein	K ($M^{-1}s^{-1} \times 10^9$)	g_{II}	$g_{\perp}$	AMT
BSOD[a]	33	5.2	1.88 ± 0.07	1.60 ± 0.04	2.260	2.088	13.8
HSOD	33	4.5	1.20 ± 0.04	0.68 ± 0.02	2.262	2.085	13.4
AFP	68	4.8	1.00 ± 0.05	0.50 ± 0.03	2.277	2.061	16.4

Molecular masses refer to the native protein. The catalytic constant (K) refers to the copper content. EPR parameters (gII, g⊥, AII) are from low-temperature X-band EPR spectra

[a] BSOD = Bovine Cu, Zn SOD; HSOD = Human Cu, Zn SOD

Table 2 Effect of different buffers and ionic strength on superoxide dismutase activity of AFP compared to the bovine and human Cu, Zn SOD

Specific activity (U/mg)[a]				
Buffer[b]	Ionic strength	BSOD[c]	HSOD	AFP
Phosphate	0.13	4.70×10^3(100)	2.60×10^3(55.3)	3.27×10^3(69.6)
HEPES	0.03	4.25×10^3(90.4)	2.85×10^3(60.6)	2.84×10^3(60.4)
HEPES + 0.1 M NaBr	0.13	4.55×10^3(96.8)	2.75×10^3(58.7)	2.73×10^3(58.1)

[a] SOD activity was determined by using the xanthine/xanthine oxidase, cytochrome c assay

[b] All buffers were at 50 mM and contained 0.1 mM EDTA. Phosphate was at pH 7.8; HEPES buffer was at pH 7.4. Values in parentheses indicate the percentage of activity based on native BSOD in phosphate buffer as 100% activity. SD in measurements was estimated to be ± 0.67

[c] BSOD = bovine Cu, Zn SOD; HSOD = human Cu, Zn SOD

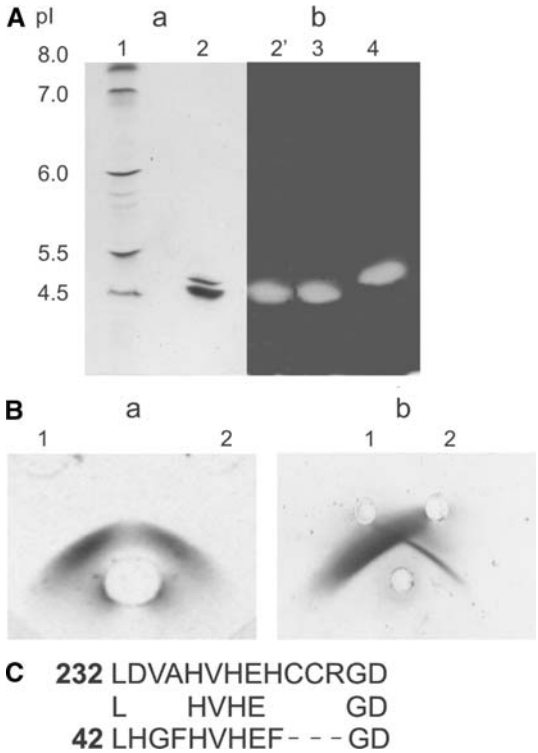

Fig. 4 A) Isoelectrofocusing analysis of AFP in the pH range 3.5–9.5. Panel a: purified AFP (lane 2); 20 μg of total protein stained with Comassie blue. Panel b: purified AFP (lane 2'), human Cu, Zn SOD (lane 3) and bovine Cu, Zn SOD (lane 4). The gel was stained for SOD activity and each lane contained 5 μg of protein. B) Ouchterlory studies. Panel a: purified AFP (well 1) and standard AFP (well 2). Panel b: purified AFP (well 1) and human Cu, Zn SOD (well 2). The monoclonal antibody against AFP was applied in the centre (2 μg/ml). C) Sequence comparison diagram showing the region of homology between human AFP and Cu, Zn SOD

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References

Aoyagi Y, Ikenaka T, Ichida F (1978) Copper (II)-binding ability of human α-Fetoprotein. Cancer Res 38:3483–3486

Beauchamp CO, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44:276–287

Bennett JA, Zhu S, Pagano-Mirarchi A et al (1998) Alpha-fetoprotein derived from a human hepatoma prevents growth of estrogen-dependent human breast cancer xenografts. Clin Cancer Res 4:2877–2884

Beyer WF, Wang Y, Fridovich I (1986) Phosphate inhibition of the copper- and zinc-containing superoxide dismutase: a re-examination. Biochemistry 25:6084–6088

Boismenu R, Semeniuk D, Murgita RA (1997) Purification and characterization of human and mouse recombinant alpha-fetoproteins expressed in *Escherichia coli*. Protein Expr Purif 10:10–26

Carrì MT, Battistoni A, Polizio F, Desideri A, Rotilio G (1994) Impaired copper binding by the H46R mutant of human Cu, Zn superoxide dismutase, involved in amyotrophic lateral sclerosis. FEBS Lett 356:314–316

Ciriolo MR, Civitareale P, Carrì MT, De Martino A, Galiazzo F, Rotilio G (1994) Purification and characterization of Ag,Zn superoxide dismutase in Saccharomyces cervisiae exposed to silver. J Biol Chem 269:25783–25787

Deutsch HF (1991) Chemistry and biology of α-fetoprotein. Adv Cancer Res 56:253–312

Deutsch HF, Taniguchi N, Evenson MA (2000) Isolation and properties of human α-fetoprotein from Hep G2 cell cultures. Tumour Biol 21:267–277

Dudich E, Semenkova L, Gobatova E et al (1998) Growth regulative activity of human alpha-fetoprotein for different types of tumor and normal cells. Tumor Biol 19:30–40

Dudich I, Tokhtamysheva N, Semenkova L et al (1999) Isolation and structural and functional characterization of two stable peptic fragments of human α-fetoprotein. Biochemistry 38:10406–10414

Ferranti P, Pucci P, Marino G et al (1995) Human α-Fetoprotein produced from Hep G2 cell line: structure and heterogeneity of the oligosaccharide moiety. J Mass Spectrum 30:632–638

Gabant P, Forrester L, Nichols J et al (2002) Alpha-fetoprotein, the major fetal serum protein, is not essential for embryonic development but is required for female fertility. Proc Natl Acad Sci USA 99:12865–12870

Goa J (1953) A micro biuret method for protein determination. Determination of total protein in cerebrospinal fluid. Scand J Clin Lab Invest 5:218–223

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage. Nature 277:680–685

Lau S, Laussac J-P, Sarkar B (1989) Synthesis and copper(II)-binding properties of the N-terminal peptide of human α-fetoprotein. Biochem J 257:745–750

Moskaleva EY, Posypanova GA, Shmyre II et al (1997) Alpha-fetoprotein-mediated targeting—a new strategy to overcome multidrug resistance of tumor cells in vitro. Cell Biol Int 21:793–799

Mizejewski GJ, Keenan JF, Setty RP (1990) Separation of the estrogen-activated growth regulatory forms of alpha-fetoprotein in mouse amniotic fluid. Biol Reprod 42:887–898

Mizejewski GJ (1997) Alpha-fetoprotein as a biologic response modifier: relevance to domain and subdomain structure. Exp Biol Med 17:333–362

Mizejewski GJ (2001) Alpha-fetoprotein structure and function: relevance to isoforms, epitopes, and conformational variants. Exp Biol Med 17:377–408

Morinaga T, Sakai M, Wegmann TG, Tamaoki T (1983) Primary structures of human α-fetoprotein and its mRNA. Proc Natl Acad Sci USA 80:4604–4608

Murgita RA, Wigzell H (1981) Regulation of immune factors in the fetus and newborn. Prog Allergy 29:54–133

Ogata A, Yamashita T, Koyama Y, Sakai M, Nishi S (1995) Suppression of experimental antigen-induced arthritis in transgenic mice producing human alpha-fetoprotein. Biochem Biophy Res Comm 213:362–366

Ouchterlony O (1967) Immunodiffusion and immunoelectrophoresis. In: Weir DM (eds) Handbook of experimental immunology. Oxford, pp 250–252

Parmelee DC, Evernson MA, Deutsch HF (1978) The presence of fatty acids in human α-fetoprotein. J Biol Chem 253:2114–2119

Pucci P, Siciliano R, Malorni A et al (1991) Human α-Fetoprotein primary structure: a mass spectroscopic study. Biochemistry 30:5061–5066

Righetti P, Drysdale JW (1971) Isoelectric focusing in polyacrylamide gels. Biochim Biophysic Acta 236:17–28

Rigo A, Viglino P, Rotilio G (1975) Polarografic determination of superoxide dismutase. Anal Biochem 68:1–8

Ruoslahti E, Pihko H, Seppala M (1974) Alpha-fetoprotein: immunochemical purification and chemical. Expression in normal state and in malignant and non-malignant. Transplant Rev 20:38–60

Ruoslahti E, Terry WD (1976) Alpha-fetoprotein and serum albumin show sequence homology. Nature 260:804–805

Steinkuhler C, Sapora O, Carrì MT, Wolfgang N, Marcocci L, Ciriolo MR, Weser U, Rotilio G (1991) Increase of Cu,Zn superoxide dismutase activity during differentiation of human K562 cells involves activation by copper of a constantly expressed copper-deficient protein. J Biol Chem 266:24580–24587

Steinkuhler C, Carrì MT, Micheli G, Knoepfel L, Weser U, Rotilio G (1994) Copper-dependent metabolism of Cu, Zn-superoxide dismutase in human K562 cells. Lack of specific transcriptional activation and accumulation of a partially inactivated enzyme. Biochem J 302:687–694

Tamano K, Sugiura M, Natsuki J et al (2005) Improvement of the lectin-antibody enzyme immunoassay of the alphafetoprotein carbohydrate chain for automation with the enzyme immunoassay robot. Biosci Biotechnol Biochem 69:1616–1619

Tomashevski AY, Narizhneva NV, Melnik TN, Uversky VN (1998) Alpha-fetoprotein structure depends on the protein purification procedure: further evidence on the structure forming role of the ligands. Prot Pept Lett 5:295–301

Tecce MF, Terrana B (1988) High-yield and high-degree purification of human α-fetoprotein produced by adaptation of the human hepatoma cell line HepG2 in a serum-free medium. Anal Biochem 169:306–311

Toder V, Bland M, Gold-Gefter L, Nebel J (1983) The effect of alpha fetoprotein on the growth of placental cells in vitro. Placenta 4:79–86

Uversky VN, Kirkitadze MD, Narizheneva NV et al (1995) Structural properties of alpha fetoprotein from human cord serum: the protein molecule at low pH possesses all the properties of the molten globule. FEBS Lett 364:165–167

Zizkovsky V, Strop P, Korcakova J, Havranova M, Mikes F (1983) Fluorescence spectroscopy and polarization, and circular dichroism in studies on pH-dependent changes in the AFP molecule. Ann NY Acad Sci USA 417:49–55