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Enzymatic and immunological activity of 4000 years aged bone alkaline phosphatase

Ulrich Weser*, Hedwig Etspüler, Yoka Kaup

Anorganische Biochemie, Physiologisch Chemisches Institut der Universität Tübingen, Hoppe-Seyler-Str. 4, D-72076 Tübingen, Germany

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Abstract Structurally intact and functionally active human bone alkaline phosphatase was isolated from clavicle fragments of IDU, an Egyptian mummy of the Old Kingdom (2150 ± 50 BC). Both anion exchange and affinity chromatographies were employed to optimise the preparation of the ancient enzyme resulting in a specific activity of 180 ± 30 mU/mg. The intactness of the bone enzyme fractions of the wheat-germ lectin affinity chromatography was successfully demonstrated in an ELISA using the monoclonal antibody BAP A. Fortunately, the mummified bone was not contaminated by fungi or bacteria.

Key words: Bone alkaline phosphatase; Ancient enzyme; ELISA; Monoclonal antibody; Egyptian mummy

1. Introduction

Amplification of ancient oligo- or polynucleotides allows the assignment of a specific human being and is a most valuable tool in molecular archaeology to ascertain the route of how the respective human population has settled [1]. Our knowledge on the mode of conservation of functionally intact enzymes is limited. Remnants or even the complete molecular architecture of ancient proteins have been repeatedly demonstrated [2]. Their functional side remains uncertain. An active core protein of Cu_2Zn_2 superoxide dismutase was obtained from air dried human brain (1200 BC) [3] and enzymic active alkaline phosphatase, a di-zinc magnesium enzyme (Fig. 1), was successfully isolated from bone samples of a ptolemeic mummy which was thoroughly pre-treated with balm components [5]. The beneficial aspect for the conservation of the alkaline phosphatase was assigned to the balm components as well as to the hydroxyl apatite bone mineral which was thought to serve as a most effective 'ion exchange matrix' for the binding of structurally intact and catalytically active proteins.

It was of interest to evaluate the time dependent limitation for the catalytic activity and the molecular architecture of the ancient alkaline phosphatase in bone samples dating back to the Old Kingdom of ancient Egypt. Clavicle fragments of a 4000-years-old Egyptian mummy called IDU (2150 ± 50 BC) were examined for both enzymically and structurally intactness. The preparation technique of the ancient enzyme was substantially improved employing both anion exchange and affinity chromatography on wheat germ lectin agarose. Proof of the unequivocal human origin of the ancient alkaline phosphatase was challenged in a specific ELISA using a monoclonal antibody directed to the human bone enzyme. This approach should strongly ascertain that the enzyme, if still detectable, can clearly be attributed to its human origin.

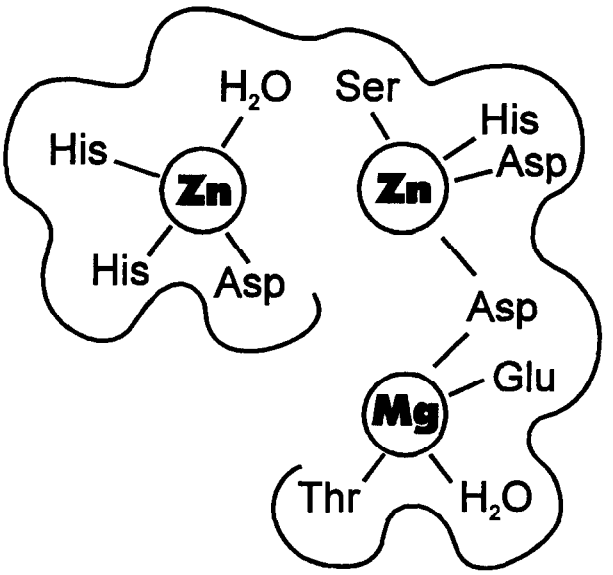

Fig. 1. Schematic view of the co-catalytic zinc binding site of *Escherichia coli* alkaline phosphatase [4].

2. Material and methods

2.1. Preparation of bone Zn_2Mg alkaline phosphatase

All steps were performed at 4°C. Clavicle fragments of IDU, an Egyptian mummy of the Old Kingdom (2150 ± 50 BC) were sandblasted using chromatographically pure aluminium oxide under sterile conditions to minimise contamination with contemporary alkaline phosphatase.

0.5 g fragments were finely ground in a porcelain mortar and suspended in 5 ml Tris buffer (pH 7.4) containing three protease inhibitors and detergent under slight agitation for 16 h as reported previously [5]. The first separation was performed by gel chromatography on a Hi-Load 16/60 Superdex 200 prep grade column equilibrated with elution buffer (20 mM Tris acetate (pH 7.4), 2 mM magnesium acetate and 0.1% (v/v) Triton X-100). Two ml fractions were collected and assayed for protein concentration and alkaline phosphatase activity at 23°C as described [5] using 1.8 mM *p*-nitrophenyl phosphate in 1 M diethanolamine and 0.5 mM magnesium acetate (pH 9.6) as substrate buffer. The active fractions were collected, concentrated and passed through an anion exchange column (Resource Q, 6.4 × 30 mm, Pharmacia) previously equilibrated with elution buffer (pH 8.2). Alkaline phosphatase was eluted with the same buffer containing 100 mM LiCl and the enzyme containing fractions were pooled, concentrated and affinity chromatographed on a wheat germ lectin agarose column (5 × 1 cm, Pharmacia). The column was initially equilibrated with elution buffer (pH 8.0) and elution was performed with 500 mM *N*-acetyl-D-glucosamine. Alternatively to the above mentioned first preparation the extract of the second preparation was gel filtrated as described above and the alkaline phosphatase containing fractions were applied directly on a wheat germ lectin agarose column.

Corresponding author. Fax: (49) (70) 7129 6391.
E-mail: akweser@mailserv. zdv. uni-tuebingen.de

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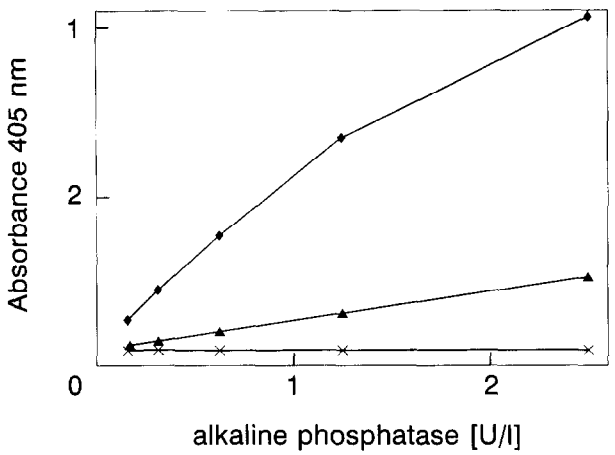

Fig. 2. Binding of monoclonal antibody BAP A to the bone extract of IDU and to the contemporary human bone alkaline phosphatase in an ELISA based on the enzymic activity of the bound antigen. ◆, contemporary enzyme; ▲, IDU; ×, control (without BAP A).

2.2. ELISA

In order to examine the intactness and the human origin of the isolated alkaline phosphatase from ancient human bone, binding to a monoclonal anti-human bone alkaline phosphatase antibody (BAP A) was tested in an enzyme-linked immunosorbent assay (ELISA) [6] based on the activity of the antibody-bound enzyme-antigen.

For the antibody production Balb/c mice were immunized with bone ALP [7]. Monoclonal antibodies BAP were produced using spleen cells which were fused with myeloma cells [8]. The obtained hybridomas were tested for antigen affinity. The positive ones were employed for the production of tumours in mice.

In ELISA all adsorption steps were performed in volumina of 100 μl each per well on a microtitre plate, the wash steps were carried out in volumina of 200 μl per well. The wells were coated with goat anti-mouse IgG (Calbiochem) (50 μg/ml) in phosphate buffer pH 6.5 (46 mM KH_2PO_4, 21 mM Na_2HPO_4) for 16 h at 23°C, then washed twice with 1.25% α-lactose monohydrate, 0.5% bovine serum albumin, 0.1% NaN_3, and once with wash buffer (50 mM Tris-HCl pH 7.5, 0.1% bovine serum albumin, 0.1% sulphobetain 14, 100 mM NaCl). The wells were incubated for 1–2 h at 23°C with BAP A (50 μg/ml) in wash buffer. After two further washes a non-immune mouse serum (Calbiochem), 40 μg/ml in wash buffer, was added to the wells for 1 h at 23°C, followed by washing as above. The coated wells were incubated with enzymically active antigen in dilution series overnight at 4°C in wash buffer. After washing a substrate buffer (10 mM *p*-nitrophenyl phosphate in 1 M diethanolamine, 0.5 mM magnesium acetate pH 9.6) was added and the formation of *p*-nitrophenol was allowed to develop for 1–2 h at 23°C. The increasing absorption at 405 nm was recorded on a microplate reader. All assays were performed in triplicate. Controls were made omitting the BAP antibody respectively the alkaline phosphatase.

3. Results and discussion

3.1. Preparation of ancient bone alkaline phosphatase

In a previous study the successful isolation of a 2300 years aged bone alkaline phosphatase being essentially identical to the contemporary enzyme was reported [5]. The time dependency on the intactness of ancient bone alkaline phosphatase was examined using portions of a clavicle taken from an Egyptian mummy called IDU (2150 ± 50 BC). During the initial gel chromatography the elution profile was essentially similar to that of the pattern obtained with the earlier ptolemeic bone extract [5]. The recovered ancient enzyme had a relative molecular mass of M_r = 190 ± 20 kDa, thus, being close to the contemporary enzyme (M_r = 200 kDa). The specific activity rose to about 40 and 57 mU/mg protein (Table 1) resembling 40 ± 7% of the activity of the ptolemeic enzyme mentioned above and 22 ± 4% to that of the contemporary enzyme. Anion exchange chromatography diminished the enzymic activity to half of the initial value. This is in contrast to the results obtained with the ptolemeic mummy extract which resulted in a 2-fold activity increase. The specific activity of the 4000 years aged alkaline phosphatase did not significantly increase using affinity chromatography on wheat germ lectin agarose. (Table 1). Thus another preparation route was devised and carried out. After gel filtration the enzymically active fractions were directly applied to an affinity column. A 4- to 5-fold enrichment in activity was detected amounting to 180 ± 30 mU/mg protein. Unlike to the catalytic activity of the ptolemeic and contemporary enzymes, which survived overnight storage at −80°C, the IDU enzyme lost its activity completely. These observations substantially support the conclusion that the alkaline phosphatase can be attributed to an ancient active protein which was safely docked for 4000 years to the apatite in the mummified bone.

As alkaline phosphatase is likewise abundant in microorganisms, control experiments were carried out to exclude the possible overlapping enzyme activity originating from microbial contamination. No anaerobic or aerobic bacteria as well as fungi could be detected in comprehensive microbial assays employing the earlier described protocol [5].

3.2. ELISA

The immunoreactivity of a monoclonal antibody (BAP A) against contemporary and ancient alkaline phosphatases was tested in an ELISA developed by *p*-nitrophenol formation in the presence of enzymically active alkaline phosphatase bound

Table 1
Alkaline phosphatase activity in extracts of clavicle fragments

	1st preparation		2nd preparation	
	Protein (μg/g bone)	Specific activity (mU/mg)	Protein (μg/g bone)	Specific activity (mU/mg)
Gel filtration	480 ± 40	57 ± 5	560 ± 30	40 ± 2
Anion exchange chromatograpy	140 ± 3	22 ± 1		
Affinity chromatography	20 ± 2	52 ± 2	15 ± 1	180 ± 30

0.5 g finely ground pelvis fragments were suspended in Tris buffer containing protease inhibitors and detergent under slight agitation for 18 h at 4 °C. During all preparation steps alkaline phosphatase activity and protein concentration [5] were assayed. The extract was gel filtrated on a Superdex 200 column (Pharmacia). In the first preparation alkaline phosphatase containing fractions were concentrated and passed through an anion exchange column. After concentration of the eluate affinity chromatography on a wheat germ lectin-agarose column was performed. The column was initially equilibrated with Tris buffer (pH 8.0) and eluted with the same buffer containing 500 mM *N*-acetyl-D-glucosamine. In the second preparation the alkaline phosphatase containing fractions of the gel filtration were applied directly to the affinity chromatography.

to the antibody. A significant immunoactivity between the ancient enzyme and the monoclonal antibody BAP A is detectable (Fig. 2). The decrease in immunoactivity of the antibody to the ancient enzyme may be due to the loss of some carbohydrate moieties, decomposition or modification of some amino acids or partial conformational change of the protein. The epitope which is recognised by BAP A is assumed to be of a marked stability which is less susceptible to age related deterioration processes of many an amino acid residue in the polypeptide chain.

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