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ALKALINE PHOSPHATASES: BIOCHEMISTRY, FUNCTIONS, AND MEASUREMENT

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Abstract
Alkaline phosphatases (ALPs) are a group of isoenzymes, situated on the external layer of the cell membrane; they catalyze the hydrolysis of organic phosphate esters present in the extracellular space. Zinc and magnesium are significant co-factors for the biological activity of these enzymes. Although ALPs are available in various body tissues and have distinct physiochemical properties, they are true isoenzymes since they catalyze a similar reaction. In the liver, ALP is cytosolic and present in the canalicular membrane of the hepatocytes. ALPs are available in placenta, ileal mucosa, kidney, bone, and liver. However, most of the ALPs in serum (over 80%) are delivered from liver and bone and in more modest quantities from the intestines. Despite the fact that alkaline phosphatases are found in numerous tissues all through the body, their exact physiological function remains largely unknown.

Keywords
Alkaline phosphatases · Mineralization · Bone alkaline phosphatase · Hypophosphatasia

Abbreviations

TNALP	Tissue non-specific
PLALP	Human placental
PIALP	Human intestinal
GCALP	Germ cell alkaline phosphatase
BALP	Bone isoform of TNSALP
LALP	Liver isoform of TNSALP
ALP	Alkaline Phosphatases
PPi	Pyrophosphate
PLP	Pyripoxal-5′-phosphate
HPLC	High-performance liquid chromatography
PTH	Parathormone
FGF23	Fibroblast growth factor 23
HA	Hydroxyapatite
5'-UTR	5 '-Untranslated region
MV	Matrix vessels
ECM	Extracellullar matrix
XLH	X -Linked hypophosphatemia
ADHR	Autosomal dominant hypophosphatemic rickets
VDRR	Vitamin D-resistant rickets
GFR	Glomerular filtration rate
HPP	Hypophosphatasia
MM	Multiple myeloma
BTMs	Bone turnover markers

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Introduction

Alkaline phosphatases (ALP, EC 3.1.3.1) are specific enzymes that, at least in vitro, can act on a wide range of substrates. They are referred to as "alkaline" because they function most effectively at pH levels above neutral, such as 8–11 [1]. ALPs are useful for a variety of biotechnological applications, including dephosphorylating phosphoproteins and DNA fragments, end-point detection in a variety of immunoassays, and as reporter molecules in vivo [2]. ALPs are abundant in nature, ranging from bacteria to all animal species, including mammals. Nonetheless, ALP activity is present in a vast array of tissues and organs. These enzymes are located primarily in the ectoplasm and are anchored to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor.

Although mammalian ALPs have broad substrate specificity and can hydrolyze or transphosphorylate a wide variety of phosphated compounds in vitro, only a few of those compounds have been confirmed to serve as natural substrates for some of the ALP isozymes (In vivo the dephosphorylation of pyrophosphate (PPi), pyripoxal-5'-phosphate (PLP), and presumably of nucleotides like ATP and proteins like osteopontin) [3].

ALPs in mammals are zinc-containing metalloenzymes encoded by multiple genes, ubiquitously expressed in multiple tissues and functioning as dimers. Enzyme activity requires three metal ions, including two Zn^{2+} and one Mg^{2+}, in the active site. Nonetheless, these metal ions also significantly influence the conformation of the ALP monomer and indirectly regulate subunit–subunit interactions [2].

ALP Expression and Structure

Four distinct genes encode distinct alkaline phosphatase isoforms in humans (Table 1). Three genes (named ALPI, ALPP, and ALPPL2) encode the tissue-specific intestinal, placental, and germ-cell ALPs, whereas the fourth gene (named ALPL and also known as TNSALP) encodes the tissue non-specific ALP (TNSALP) that is abundantly expressed in the skeleton, liver, kidney, and developing teeth. The amino acid sequences of intestinal, placental, germ-cell, and tissue non-specific ALPs are distinct, making them true isoenzymes. TNSALP is subject to post-translational modifications, and the carbohydrate composition of bone and liver ALP is distinct. Since the amino acid sequences of these two products are identical, they are not true isoenzymes but rather isoforms of TNSALP. The first three isoenzymes have a high degree of homology (90–98%), their genes are clustered on the long arm of chromosome 2 (bands q34–q37.1), and their structures are virtually identical. The structural similarity of these three genes indicates their divergent evolution from a common ancestor. The fourth ALP, TNSALP, is 50 percent identical to the other three, but it is at least five times larger, primarily due to differences in intron size. The ALPL gene is located on chromosome 1's short arm (bands p34–36.1). Bone and liver isoforms of TNSALP are formed through post-translational modifications of the ALPL gene product (specifically through differences in carbohydrate composition). They are highly expressed in the liver,

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bone, and kidney, as well as at lower levels in a variety of other tissues (e.g., developing cerebral cortex, spinal cord) [4].

All ALP isoenzymes function as homodimers and are attached to the outer cell membrane by a glycophosphatidyl-inositol (GPI) anchor. The release of soluble ALP into the systemic circulation is achieved through cleavage by a specific phospholipase D [5].

Each monomer (molecular weight: 66 kDa) is transferred to the endoplasmic reticulum, where carbohydrate chains are added as O- and N-linked sugar chains. The modified protein is then processed in the Golgi apparatus and ultimately localized on the outer cell membrane by means of the GPI anchor. The two tissue-specific isoforms of TNSALP (liver- and bone-specific isoforms) have distinct sugar chains due primarily to differences in O-linked glycosylation. [2].

Besides these two main isoforms, four isoforms of bone alkaline phosphatase (BALP) and three isoforms of liver alkaline phosphatase (LALP) have been identified by Highperformance liquid chromatography (HPLC) [6, 7].

The amino acid sequence of human TNSALP molecule is 57% identical and 74% homologous with the human placental ALP (PLALP) molecule.

ALP Function in Bone Mineralization

Approximately 90 years ago, Robinson was the first to recognize the important role of ALP in bone formation and suggested that this enzyme functioned in skeletal mineralization by liberating inorganic phosphate (Pi) for hydroxyapatite (HA) propagation [8]. The high levels of ALP expression was noted and the first hypothesis linked ALP to hard tissue formation. In this hypothesis ALP was postulated to increase the local concentration of inorganic phosphate, a concept known as the 'booster hypothesis.' As our knowledge about the biology of hard tissues and mineral metabolism has been expanded, the role of ALP as a marker for osteogenic activity has been consistently solidified. On the other hand, the detailed mechanism of how ALP functions in hard tissues is still considered controversial. However its crucial role is now undisputed since it became apparent that the mutations in the gene coding for TNSALP can lead to genetic diseases known as hypophosphatasia (see below for details).

Table 1 Summary of the gene nomenclature, accession numbers, common names, tissue distribution, and function, if known, for the human and mouse alkaline phosphatase isozymes

Human genes	Common names, synonyms	Tissue distribution	Function
ALPL (TNSALP)	Tissue non-specific alkaline phosphatase; TNSALP	Developing nervous system, skeletal tissues, liver, kidney	Bone mineralization
ALPP	Placental alkaline phosphatase; PLALP	Syncytiotrophoblast, a variety of tumors	Unknown
ALPP2	Germ cell alkaline phosphatase, GCALP	Testis, malignant trophoblasts, testicular cancer	Unknown
ALPI	Intestinal alkaline phosphatase, IALP	gut, influenced by fat feeding and ABO status	Intestinal absorption

TNSALP is implicated in the physiological mineralization of hard tissue where it hydrolyzes pyrophosphate to provide inorganic phosphate necessary to promote mineralization [1,9]. Mineralization is a tightly controlled process by which minerals are deposited within out outside the cells of a variety of organisms. It is dependent on the complex interplay between inorganic ions [Ca^{2+},

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Pi and PPi], hormones [such as parathormone (PTH) and fibroblast growth factor 23 (FGF23)], and enzymes [mainly TNSALP].

In humans (as in most vertebrates) the deposited minerals are composed of hydroxyapatite (HA) and are found in specific areas of the extracellular matrix (ECM). Physiological mineralization occurs by series of physicochemical and biochemical processes in hard tissues, such as bone growthplate cartilage and teeth, in which several tissue-specific cells are responsible for the mineralization [9, 10].

In growth-plate cartilage, mineralization occurs in the hypertrophic zone, where late proliferative and early hypertrophic chondrocytes are responsible for HA formation [11]. In bone, osteoblasts and matrix vesicles (MV) are key players for mineralization. The osteoblasts are lining the osteoid and are responsible for HA formation, whereas in teeth odontoblasts share this responsibility. MVs, in which hydroxyapatite crystals are formed, are extracellular vesicles (50–200 nm) formed by polarized budding from the surface membrane of osteoblasts, chondrocytes, or odontoblasts.

In contrast, pathological mineralization is responsible for calcification of soft tissue. Uncontrolled or pathological mineralization can occur in any soft tissue among which cardiovascular tissue and the kidneys (for more details see below) [12]

Tissue Non-Specific ALP in Disease

Increased levels of alkaline phosphatases activity can be found in human serum during either physiological or pathological processes that correspond to a high osteoblastic activity [13]. In healthy humans, serum BALP activity is increased during skeletal growth or fracture healing [14, 15]. However, several diseases are also characterized by elevated BALP activity, such as Paget's disease, rickets and osteomalacia, osteoporosis (particularly after fractures), neoplastic diseases, including osteosarcoma and metastatic bone diseases, and primary and secondary hyperparathyroidism.

In contrast, decreased total ALP activity is rarely found in human serum but can occur in hypophosphatasia, in multiple myeloma with osteolytic lesions, secondary to growth hormone deficiency or in hypoparathyroidism. The cause of variation of alkaline phosphatases in various diseases is presented in Table 2 and the generally accepted reference ranges are presented in Table 3.

Recently, several studies have reported an association between increased ALP or BALP concentrations and increased morbidity and mortality in patients suffering from advanced CKD [16–20].

The Measurement of ALPs

MEASURING TOTAL ALP ACTIVITY

The measurement of total ALP activity in serum or plasma remains one of the most widely employed tests in clinical laboratories. ALPs hydrolyze a large variety of organic monophosphate esters with formation of an alcohol or phenol and a phosphate ion. Several methods have been described over the

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years based on the use of different substrates and different assay conditions (i.e., pH, buffers, reaction temperature) [76]. Currently, most tests used today on automated clinical chemistry analysers that measure the total ALP activity in serum use conventional photometric assay with the following reactions: ALP catalyzes the hydrolysis of p-nitrophenylphosphate (p-NPP), which is colorless, to phosphate and free p-nitrophenol (p-NP). Under alkaline conditions p-NP is converted to 4-nitrophenoxide, which has intense yellow color and absorbs at 405 nm.

The IFCC Committee on Reference Systems of Enzymes (C-RSE) has proposed in 2011 a Reference procedure for the measurement of alkaline phosphatase [77]. This work was based on a previous proposal by an IFCC work [78, 79] but differs by the temperature (37 °C vs. 30 °C, respectively). In the standardization of enzyme assays, a Reference measurement procedure, which defines the conditions under which a given enzyme activity is measured, occupies the same role as that of primary reference materials for nonenzyme analytes of known mass or substance concentration. Thus, to prevent errors, it is extremely important to select and define carefully designed recommended procedures for each enzyme determination. To achieve a complete standardization, the components of the reaction system (e.g., pH and buffer, temperature, presence of activators and inhibitors, substrate nature, and concentration) need to be carefully respected by all the analytical systems. If this is not the case, the magnitude of the measured activity will also change. Consequently, two procedures that measure the catalytic activity of the same enzyme but under different analytical conditions may produce different results for a given sample thus preventing the use of the same reference intervals or decision limits.

Table 2 Main causes of clinical variations of alkaline phosphatases

Disease	Cause of the change of Alkaline Phosphatases	Variation	References
Bone disorders			
Rickets	Increased osteoblastic activity	Increased	[21–23]
Paget's disease	Increased osteoblastic activity	Increased	[24]
Osteomalacia	Increased bone remodelling	Increased	[25, 26]
Osteoporosis	High bone turnover/fractures	Normal/increased	[27]
Treatment of osteoporosis		Increased or decreased depending on therapy	[27–30]
Primary or secondary hyperparathyroidism,	Bone origin/ Increased bone remodelling	High normal/increased	[31–33]
Fanconi syndrome	Chronic hypophosphatemia leading to osteomalacia	Increased	[34, 35]
Bone cancer, osteosarcoma and bone metastasis	Increased osteoblastic activity/tumor cells	Increased	[36–40]
Hepatobiliary diseases			
Extrahepatic biliary obstruction (Choledocholithiasis / malignant obstruction)	New synthesis in the hepatocytes adjacent to the biliary canaliculi	Increased	[41, 42]
Intrahepatic biliary obstruction	Liver origin	Increased	[41, 42]
Liver Cirrhosis	Liver origin	Increased	[41, 42]
Viral hepatitis	Liver origin	Increased	[42]
Alcoholic, non-alcoholic, and drug induced hepatitis	Liver origin	Increased	
Primary malignancy (primary hepatocellular carcinoma)		Increased	
Liver metastasis		Increased	
Non-alcoholic fatty liver disease (NAFLD)	Liver production	Increased	
Infiltrative diseases (Lymphoma, Amyloidosis)	Liver production	Increased	
Wilson's disease	Liver production	Transient decrease	[43, 44]
Granulomatous diseases (sarcoidosis, tuberculosis, etc.)	Liver origin (elevated alkaline phosphatase may be more common in patients with granulomas related to sarcoidosis or PBC or drug-induced injury due to bile ductule involvement	Increased	[45]
Drug induced ALP increase (including total parenteral nutrition)	Intrahepatic Cholestasis / enzyme induction	Variable increase depending on drug and duration of therapy	[46, 47]

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Table 2 (continued)

Disease	Cause of the change of Alkaline Phosphatases	Variation	References
Other			
Pregnancy	Placental origin	Slight increase	[48]
Hypophosphatasia	Decreased activity due to the mutations in the TNSALP gene	Decreased	[4, 49]
Paraneoplasic activity	Regan enzyme (placental isoform)	Increased	
Physiologic bone growth	Increased osteoblastic activity	Increased	[9]
Healing of bone fractures	Increased osteoblastic activity	Increased	[50, 51]
Drug induced low ALP	Osteoblast inhibition	Variable decrease again depending on drug and duration of therapy	
Endocrine disorders			
Cushing syndrome	Decrease in osteoblast number and function	Decreased	[52–54]
Hypothyroidism	Bone remodeling cycle is prolonged in hypothyroidism	Decreased	[55–58]
Hyperthyroidism	Both liver and bone origin / Elevation in ALP is due to increased osteoblastic activity and driven mainly by bone isoenzyme	Increased	[59–61]
Hematological diseases			
Multiple myeloma	Osteoblast Inhibition	Normal/Decreased	[62, 63]
Sickle cell disease	Liver production/sickle cell intrahepatic cholestasis	Increased	[64–66]
Diet			
Malnutrition, starvation	Insufficient activation of the Enzyme	Decreased	[67]
Magnesium and Zinc deficiency	Insufficient activation of the Enzyme	Decreased	[68]
Vitamin D deficiency	in the resorption phase, low osteoblast activity and low release of BALP stimulate 25-OHase activity; on the contrary, higher BALP levels of the formation phase inhibit the activity of CYP2R1	Normal/Increased	[69–72]
Vitamin C deficiency		Decreased	[73, 74]
Vitamin B12 deficiency	Diminished synthesis by osteoblasts	Decreased	[75]

Unfortunately, IFCC's reference measurement procedure is not always used in all clinical laboratories. A study performed in Italy in 2016 showed "a substantially poor comparability" among the clinical laboratories that participated using several commercial ALP measuring systems [80]. The availability of an internationally agreed reference system does neither automatically mean that the traceability to it is immediately implemented. For instance, until 2020, medical laboratories in Japan utilized the Japan Society of Clinical Chemistry (JSCC) method for blood ALP catalytic concentration measurement. The JSCC and IFCC methods differ in terms of the type of buffer solution used [81]. This difference results in the IFCC method yielding human blood ALP estimates at levels of approximately one-third more than those of the JSCC method [82]. Hence, the measured values of Alkaline phosphatase in Japan vs. the rest of the world cannot be used interchangeably and, to the best of our knowledge, no recent publication (in English, at least) has shown the opposite.

At this stage, it is important to emphasize the need of well-designed external quality assessment schemes in which the target values of ALP should be assigned with the IFCC RMP and not based on consensus values (such as peer-group means or other indicators of central tendency) and quality specifications indicators derived from non-objective models [83–85]. Such schemes may fail to highlight analytical problems and can lead to misleading conclusions of false security. Yet, a new study that will evaluate the trueness of serum total ALP activity measurement in clinical laboratories worldwide using the above-mentioned standards seems necessary.

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If these enzymatic assays provide useful information about the activity of total ALP in human serum, they cannot discriminate the various ALP isoenzymes and isoforms. Therefore, total ALP used as a marker of bone formation may be limited in patients with hepatic disorders [86]. In order to obtain better specificity and selectivity, immunoassays that were able to quantify the bone isoform of ALP (BALP) were developed and are now used in clinical laboratories [87, 88].

SEPARATION AND QUANTIFICATION OF ALP ISOENZYMES - MEASUREMENT OF THE BONE ISOFORM OF TNSALP

The challenge to BALP quantification in human serum is to differentiate it from liver isoform (LALP). Since they share the same amino acid sequence and that their differences are limited only to the rate of post-translational glycosylations. Various electrophoretic techniques that exploit these carbohydrate differences have been developed and used over the years to specifically measure the bone isoform. Such methods need a sample pre-treatment (most of the time with lectin) to separate the bone from the liver forms. Knowing the total ALP activity of the sample, the bone isoform activity (as well as the other isoforms' activities) can thus be calculated. Such techniques are not however routinely performed in every lab since they require an important expertise as well as dedicated instruments and reagents. Immunoassays using either highly specific monoclonal or polyclonal antibodies for bone-ALP in various formats have allowed more widespread use in clinical chemistry laboratories.

The first immunoassay for BAP was an immunoradiometric assay (IRMA) developed by Hybritech under the commercial name Tandem-R Ostase [89, 90]. This assay involved two specific murine monoclonal antibodies directed against different antigenic sites on bone-ALP.

The assay was calibrated using calibrators made of purified bone-ALP extracted from osteosarcoma line cells (SAOS-2 cells). The assay measured the mass of boneALP and results were expressed in ng/mL [91, 92]. This assay used radioisotope-labeled reagents and required overnight incubation. Evaluation studies showed that cross reactivity with the liver isoform with this assay ranged between 14.3 and 18.3% [89, 90, 93, 94]. In several studies this assay showed promise as an aid in monitoring and diagnosis bone diseases associated with increased osteoblastic activity [89, 95–97].

The second assay was an immunocapture enzyme immunoassay developed by Gomez et al., in 1995 [98]. This assay was using a monoclonal antibody prepared on mice immunized with ALP extracted and purified from SAOS-2 cells [99]. This assay was measuring the activity of the bone isoform that was captured on the solid phase and the results were expressed in U/L where 1 U represents the enzyme activity which hydrolyzed 1 µmol of p-NPP per min at 25 °C. Calibrators were made of ALP produced from SAOS-2 cells. This assay was commercialized by Metra Biosystems as Alkphase-B [100]. The results reported after the comparison of these two assays were variable [94, 101, 102].

In 1998 a new commercial enzyme immunoassay was marketed under the name Tandem-MP Ostase and was claiming to measure the bone isoform of ALP. This was an immunocapture ELISA using a single monoclonal antibody. Based on a similar principle with the previous described immunoassay, this assay was also measuring the activity of the captured bone isoform [87]. However, calibration of this assay

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was established to match the calibration of the IRMA Tandem-R Ostase. If calibrators were also produced using purified ALP extracted from SAOS-2 cells, the value of these calibrators was given in µg/L. Hence, the activity value of the calibrators transformed into mass values in order to match the immunometric assay results [87].

Today, the immunoradiometric (IRMA) assay is produced and distributed by Beckman Coulter. In its present form, produced in the Czech Republic, the assay uses coated tubes with the first antibody, whereas the Hybritech version used coated plastic beads.

Different automated methods for BAP measurement are proposed by Beckman Coulter (Access Ostase), IDS (iSYS Ostase), and DiaSorin (Liaison Ostase), this latter using two monoclonal antibodies specific for BAP in a "sandwich" feature [103], whereas the two other methods are one-step immunoenzymatic assay using a single monoclonal antibody specific to BAP. The characteristics of all commercially available assays today are shown in Table 4.

Table 4 Current commercially available assays measuring the bone isoform of ALP

Method	Measures	Calibration against	Origin of the antibodies	Origin of the antigen	Claimed CV
Beckman Coulter Ostase IRMA	Mass of the enzyme. Results in µg/L	Hybritech Tandem-R Ostase	Beckman Coulter	Beckman Coulter	< 13.6%
Beckman Coulter Ostase Access	Activity of the enzyme Results in mass (µg/L) after calibration	Hybritech Tandem-R Ostase	Beckman Coulter	Beckman Coulter	< 6.5%
IDS iSYS Ostase	Activity of the enzyme. Results in mass (µg/L) after calibration	Beckman Coulter Ostase Access	Beckman Coulter	Beckman Coulter	< 9%
IDS Ostase BAP EIA	Activity of the enzyme. Results in mass (µg/L) after calibration	Beckman Coulter Ostase Access	Beckman Coulter	Beckman Coulter	< 6.4%
DiaSorin Liaison Ostase	Mass of the enzyme. Results in µg/L	Beckman Coulter Ostase Access	Beckman Coulter Ostase Access for the capture antibody, DiaSorin for the second, isoluminol bound, antibody	Beckman Coulter, DiaSorin	< 8.1%
Quidel MicroVue	Activity of the enzyme. Results in U/L Divide by 0.488 to yield µg/L	1 unit of BAP is defined as 1 µmol of p-nitro-phenylphosphate hydrolyzed per minute at 25 °C	Quidel (Metra)	Quidel (Metra)	< 7.6%

Conclusion

Although ALP is one of the most thoroughly studied enzymes, its metabolism has not yet been fully elucidated. The diagnostic value of routine measurement of serum total ALP activity has been firmly established and it became one of the most useful laboratory tests to assess cholestasis and bone disease.

TNSALP is a key enzyme responsible for skeletal tissue mineralization. It is involved in the dephosphorylation of various physiological substrates and has vital physiological functions, including extra-skeletal functions (is implicated in ectopic pathological calcification of soft tissues, especially the

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vasculature). Although it is the crucial enzyme in mineralization of skeletal and dental tissues, it seems a logical clinical target to attenuate vascular calcification.

Enzymatic assays that measure total ALP activity provide information about the level of total ALP in human serum, but they cannot discriminate the various ALP isozymes and isoforms. Although standardization of these assays has been achieved by IFCC their use and the reliability in the clinical assessment of bone formation is limited in patients with hepatic disorders. However these assays remain a primary tool in the diagnosis of different diseases involving bone metabolism.

Immunoassays that claim to measure the bone isoform of TNSALP (BALP) have also limitations and a considerable inter-method variability [104]. These discrepancies are probably caused by a lack of standardization related to the lack of a reference method and the absence of an international standard for BALP. Standardization of these assays is a prime target of the IFCC committee on bone metabolism.

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