Differences in Sialic Acid Residues Among Bone Alkaline Phosphatase Isoforms: A Physical, Biochemical, and Immunological Characterization

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Received: 3 December 2001 / Accepted: 28 March 2002 / Online publication: 18 September 2002

Abstract. High-performance liquid chromatography (HPLC) separates three human bone alkaline phosphatase (BALP) isoforms in serum; two major BALP isoforms, B1 and B2, and a minor fraction, B/I, which is composed on average of 70% bone and 30% intestinal ALP. The current studies were intended to identify an in vitro source of the BALP isoforms for physical, biochemical, and immunological characterizations. The three BALP isoforms were identified in extracts of human osteosarcoma (SaOS-2) cells, by HPLC, after separation by anion-exchange chromatography. All three BALP isoforms were similar with respect to freeze-thaw stability, solubility, heat inactivation, and inhibition by L-phenylalanine, L-homoarginine, and levamisole. The isoforms were also kinetically similar (i.e., maximal velocity and $K_M$ at pH 8.8 and pH 10.0). The isoforms differed, however, with respect to sensitivity to precipitation with wheat germ agglutinin (WGA), $P < 0.001$, but not Concanavalin A. At 3.0 mg/ml, WGA precipitated approximately 25% of B/I but more than 80% of B1 and B2. Molecular weights were estimated by native gradient gel electrophoresis: B/I, 126 kDa; B1, 136 kDa; and B2, 141 kDa. Desialylation with neuraminidase reduced the apparent sizes of B1 and B2 to 127 kDa (i.e., approximately to that of B/I). The total carbohydrate content was calculated to be 18 kDa, 28 kDa, and 33 kDa (i.e., 14%, 21%, and 23%) for the BALP isoforms, B/I, B1, and B2, respectively. The number of sialic acid residues was estimated to be 29 and 45, for each B1 and B2 homodimer, respectively. Apparent discrepancies between these estimates of molecular weight and estimates based on gel-filtration chromatography were attributed to nonspecific interactions between carbohydrate residues and the gel filtration beads. All three BALP isoforms showed similar dose-dependant linearity in the commercial Alkphase-B and Tandem-MP Ostase immunoassays, $r = 0.944$ and $r = 0.985$, respectively ($P < 0.001$). In summary, our data indicate that B1 and B2 have more (or more reactive) sialic acid residues compared with B/I, which mainly explains the apparent differences in molecular weight. Future investigations will focus on the clinical and functional significance of the revealed differences in sialic acid residues.

Key words: Biochemical markers — Glycosylation — Osteoblasts — Kinetics — Immunoassay

An increasing number of biochemical markers are now available for studies of bone turnover, metabolic bone disease, and for monitoring response of treatment regimes in osteoporosis [1]. Alkaline phosphatase (ALP) (EC 3.1.3.1) is one of the most used biochemical markers of osteoblastic bone formation. Approximately 95% of the total ALP activity in human serum is derived from bone and liver sources which occur in a ratio of approximately 1:1 in healthy adults [2]. Both bone ALP (BALP) and liver ALP (LALP) are encoded by the tissue nonspecific ALP gene locus and are referred to as isoforms of the same isoenzyme. BALP is a glycoprotein and functions as an ectoenzyme attached to the osteoblast cell membrane by a glycosylphosphatidylinositol (GPI) anchor [3, 4]. BALP has been reported to be necessary for the initiation of mineralization by osteoblast-derived matrix vesicles but not for the continuation of the processes [5–7], however, the precise function of BALP has still to be elucidated [8].

Little is known about the specific carbohydrate structures present in the ALP isoenzymes; however, one difference in carbohydrate structure between the ALP isoenzymes is that intestinal ALP does not contain sialic acid, but BALP, LALP, and placental ALP do [9, 10]. This difference is thought to contribute to the large differences in half-lives in the circulation for different ALPs, e.g., intestinal ALP (7.5 h) [9]; BALP (2 days) [11]; and placental ALP (7 days) [12]. The amino acid sequence of the tissue nonspecific ALP gene suggests five putative N-glycosylation sites in BALP and LALP and, recently, Nosjean et al. [13] confirmed that both BALP and LALP are N-glycosylated; however, they did not report the number of N-glycosylation sites. Their data also suggested that the main difference between BALP and LALP is due to a difference in O-glycosylation (i.e.,
BALP bears some O-linked carbohydrates whereas LALP does not [13]. Finally, it has also been reported that BALP has more fucose [14] and sialic acid residues [13] compared with LALP. Nevertheless, the specific structures of the N-linked and O-linked oligosaccharides have not been reported.

We have utilized a previously described weak anion-exchange high-performance liquid chromatography (HPLC) method that can resolve at least six different ALP isoforms in serum from healthy adults: three BALP (B/I, B1, and B2) and three LALP (L1, L2, and L3) isoforms [15, 16]. In healthy adults, the three BALP isoforms, B/I, B1, and B2, account on average for 4, 16, and 37%, respectively, of the total serum ALP activity. In serum, the minor fraction B/I is not a pure BALP isoform as it co-elutes with intestinal ALP and is composed, on average, of bone (70%) and intestinal (30%) ALP. The circulating levels of these BALP isoforms can vary independently during the pubertal growth spurt [17] and in several disease states such as growth hormone deficiency [2], hypophosphatasia, X-linked hypophosphatemia, stress fractures [15, 18], metastatic bone disease [19], Paget’s bone disease [20], celiac disease [21], and chronic renal failure [22]. Some anatomical differences have previously been reported; cortical bone had approximately two-fold higher activity of B1 compared with B2 and, conversely, B2 was approximately two-fold higher in trabecular bone compared with B1 [23]. Recently, a fourth BALP isoform (B1x) was discovered and characterized in bone tissue and in serum from patients with severe renal insufficiency and on chronic dialysis therapy [22, 23].

The current study was intended to characterize, by physical and biochemical means, the three BALP isoforms previously found in serum samples of healthy children and adults. Since the BALP isoforms all represent the same gene product (i.e., they have the same amino acid composition), we postulated that the structural differences between the BALP isoforms, which allow for HPLC separation, may reflect different patterns of glycosylation. In addition, we also investigated the cross-reactivity of the various BALP isoforms with two commercially available immunoassays of BALP. We used human osteosarcoma (SaOS-2) cells for these studies because their BALP activity is structurally, immunologically, and kinetically indistinguishable from normal human BALP activity [24–27]. Furthermore, previous studies have shown that human BALP standards prepared from SaOS-2 cells contain the same BALP isoforms (B/I, B1, and B2) as observed in the circulation [22]. Finally, we chose SaOS-2 cells as a possible BALP isoform source because we wanted to prepare significant and sufficient amounts for characterization of these isoforms. Since SaOS-2 cells contain BALP in amounts on the order of 1 U/mg cell protein [24–27], we presumed that they would be a suitable source.