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A comparative study of 5'nucleotidase and alkaline phosphatase in human placenta during development

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Abstract. Activities and a few properties of alkaline phosphatase and 5′-nucleotidase were compared in the developing human placenta. Both the enzymes were mostly membrane-bound and displayed similar developmental patterns with the highest activities at 24/26 weeks of the placenta. L-Phenylalanine, L-tryptophan and L-leucine were inhibitors of alkaline phosphatase, whereas they had no effect on the 5′-nucleotidase. Alkaline phosphatase from a late stage of gestation appeared to be almost heat-stable. An appreciable part of 5′-nucleotidase was also resistant to heat inactivation and this fraction varied with gestational age of the tissue. For both the enzymes, V_{max} changed without altering K_m values with periods of gestation. Ca^{2+} , Mg^{2+} and Mn^{2+} ions stimulated the alkaline phosphatase activity and Hg^{2+} , Zn^{2+} , Cu^{2+} ,Ni^{2+} were inhibitory. 5′-Nucleotidase was not activated by any of these cations. EDTA and Concanavalin A inhibited both the enzymes, although the extent of inhibition was different and also varied with gestation.

Keywords. Human placenta; alkaline phosphatase; 5′-nucleotidase.

Introduction

5′-Nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) is widely distributed in living systems (Bodansky and Schwartz, 1968) including human placental tissues (Hayashi *et al*., 1964: Krishnakantha and Maguir, 1978). The presence of phosphomonoesterases with peak activity at alkaline pH (Orthophosphoric monoester phosphohydrolase, EC 3.1.3 .1) is a distinguishing feature of syncytial trophoblast of the placenta (Fishman *et al*., 1976; Sugiura *et al*., 1977). The activity of non-specific phosphatases interfered with the evaluation of 5′-nucleotidase activity (Bodansky and Schwartz, 1968). In an attempt to circumvent this difficulty, it seemed worthwhile to study the properties of these two enzymes in human placental tissues. We have, therefore, conducted a comparative study of 5′-nucleotidase and alkaline phosphatase throughout the gestational ages of human placenta and a method for assaying 5′-nucleotidase is reported.

Materials and methods

Chemicals

p-Nitrophenyl phosphate, 5′ AMP and bovine serum albumin were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Concanavalin A was a gift from Dr B. K. Bachhawat, Indian Institute of Experimental Medicine, Calcutta. All other chemicals used were of analytical grade and were purchased locally.

Biological Material

Human placental tissues were collected from the Department of Obstetrics and Gynaecology, S.S.K.M. Hospital, Calcutta. Term placentas were obtained from normal term deliveries and other tissues of different getational ages were collected from women undergoing legal abortion either by suction or by hysterotomy. Gestational age was determined from the period of amenorrhea and by the size, weight and crown-rump length of the fetus. This method generally provides data correct to ± one week (Kaplay, 1976). The placenta were kept on ice until processing.

Preparation of the enzymes

The cross-sectional pieces of the tissues were taken, chopped and washed with ice-cold distilled water to free it from blood as far as practicable. The tissues were homogenized in a Potter-Elvehjem glass homogenizer at 0-4° C in 0.25 M sucrose to make a 10% (w/v) tissue concentration. The cell debris and nuclear residues were removed by centrifugation in the cold (0-4°C) at 1500 *g* for 10 min in an International Refrigerated centrifuge (Model B-20). The supernatant fluid was then recentrifuged at 12,000 *g* for 30 min for isolation of the mitochondria. The supernatant thus obtained was centrifuged at 105,000 *g* for 1 h in a Spinco Ultracentrifuge, Model L. The pellet was used as the microsomal fraction and the supernatant as the cytosol fraction. The microsomal pellet was suspended in ice-cold 0.25 M sucrose. This suspension, unless otherwise stated, was used as the enzyme preparation for the study of 5′-nucleotidase and alkaline phosphatase.

Enzyme assay

5′-Nucleotidase activity was measured in a reaction mixture containing 80 mM Tris-HC1, pH 7.5; 0.5 mM 5' AMP, 30-40 μg of the enzyme and with or without 20 mM L-tryptophan (a potent inhibitor of alkaline phosphatase) in a total volume of 1.0 ml. Following a 30 min incubation at 37°C, 0.25 ml of ice-cold 30% trichloroacetic acid was added. The samples were then chilled in ice and centrifuged for 10 min at 2000 *g*. The phosphate content (P_i) in the supernatant was determined by the method of Lowry and Lopez (1946). The amount of P_i liberated/mg protein/h was considered as the specific activity of 5′-nucleotidase.

For the estimation of alkaline phosphatase, the assay mixture containing 1 mM of *p*-nitrophenylphosphate, 80 mM Tris-HCl, pH 9.6 and 30-40 μg of the enzyme preparation was incubated at 37°C for 30 min. The reaction was stopped by the addition of 0.1 N NaOH and the formation of *p*-nitrophenol was measured colorimetrically in a Spectronic 20 spectrophotometer at 420 nm. The specific activity of alkaline phosphatase was defined as the amount of *p*-nitrophenol liberated/mg protein/h.

Estimation of protein

Protein was estimated by the method of Lowry *et al*., (1951) using crystalline bovine serum albumin as the Standard.

Results

Table 1 summarizes the enzyme activities in the microsomal and supernatant fractions of human placenta with increasing periods of gestation. Both the enzymes were detected in human placenta as early as the 6th week of gestation. The enzyme

Table 1. Activity of alkaline phosphatase and 5'-nucleotidase in human placenta with increasing periods of gestation.

Gestation (weeks)	5′-nucleotidase [a] ($\times 10^{-4}$)		Alkaline phosphatase [b] ($\times 10^{4}$)	
	Microsome	Cytosol	Microsome	Cytosol
6-8	12.59±1.02	0.86±0.08	1.02±0.12	0.26±0.03
10-12	15.92±1.10	1.31±0.10	3.00±0.17	0.35±0.03
14-16	18.02±1.39	1.42±0.11	4.05±0.20	0.59±0.06
18-20	21.97±1.63	1.64±0.13	8.17±0.83	1.40±0.12
22-26	23.40±1.98	2.12±0.19	15.73±1.21	3.00±0.16
36-40	20.79±1.60	2.65±0.20	14.91±0.98	3.66±0.28

The results are mean ± SEM of 6 observations in each case.

[a] Specific activity of 5'-nucleotidase is expressed as μ mol P_i liberated/mg protein/h.

[b] Specific activity of alkaline phosphatase expressed as μ mol *p*-nitrophenol liberated/ mg protein/h.

activities in the microsomal fraction increased with the gestational age, until 24/26 weeks (the latest stage studied). The activities, however, slightly declined at term. The supernatant fraction maintained a steady increase in activity with the advancement of pregnancy.

Table 2 compares the extent of inhibition of placental alkaline phosphatase and 5′-nucleotidase by L-phenylalanine, L-tyrosine and L-leucine. Alkaline phosphatase was strongly inhibited by these amino acids and the sensitivity gradually decreased with placental maturity. On the other hand, these amino acids had no effect on 5′-nucleotidase activity and this property was utilized in determining placental 5′-nucleotidase activity in the present study.

Table 2. Effect of amino acids on alkaline phosphatase and 5′-nucleotidase from the developing human placenta.

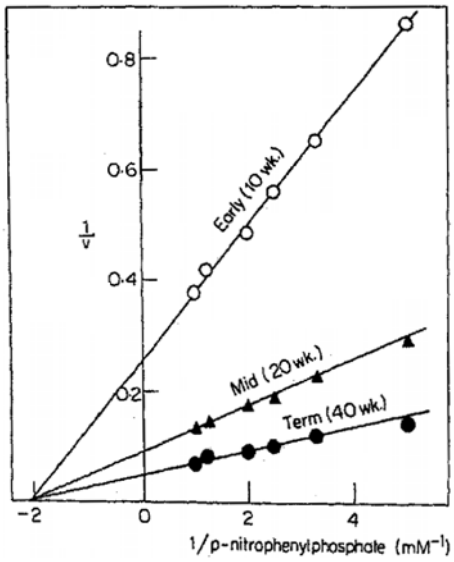
Amino acid used (mM)	inhibition (%)		
	Early (10 week)	Mid (20 week)	Term (40 week)
L-Phenylalanine (1)	45 (1)	26 (1)	19 (0)
L-Phenylalanine (5)	77 (2)	64 (2)	56 (2)
L-Tryptophan (1)	54 (2)	36 (1)	29 (1)
L-Tryptophan (5)	90 (2)	77 (2)	70 (2)
L-Leucine (1)	29 (0)	20 (0)	14 (0)
L-Leucine (1)	60 (1)	47 (1)	40 (0)

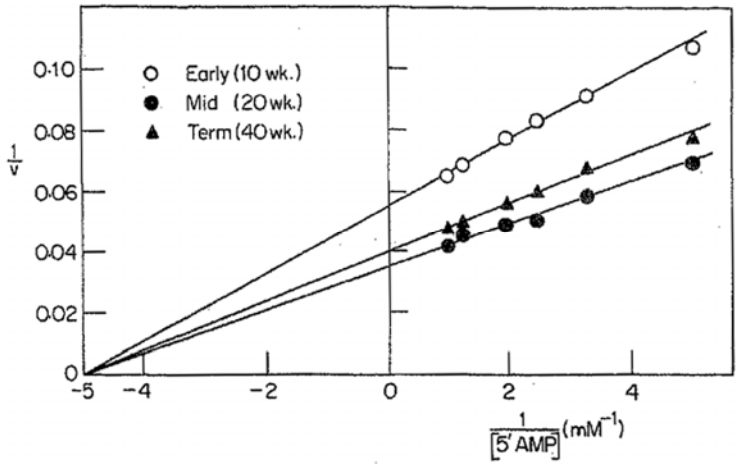
The incubation medium contained L-amino acid (1 mM or 5 mM) and the control system contained the corresponding D-amino acid. Percentage inhibition was calculated from (D–L)×100/D

Figures in parenthesis indicate the percentage inhibition of 5′-nucleotidase.

The results are the average of 3 observations for each gestation period.

Lineweaver-Burk plots for alkaline phosphatase and 5′-nucleotidase during different stages of gestation are shown in figures 1 and 2 respectively. In both the cases, K_m values remained constant, while V_{max} changed during placental development.





Figures 1 and 2. Double reciprocal plots of the velocity against substrate concentration in different gestation of placenta. v–represents velocity expressed as specific activity.

Table 3 presents the kinetic parameters of *p*-nitrophenylphosphate hydrolysis in the absence and presence of inhibitors. The kinetic studies revealed that these amino acids altered both the K_m and V_{max} values of *p*-nitrophenylphosphate hydrolysis throughout the gestational ages.

Table 3. Kinetic parameters of alkaline phosphatase with inhibitors.

Treatment (mM)	K_m (mM)	V_{max}[a] × 10^4	K_m (mM)	V_{max}[a] × 10^4
	Early gestation (10 weeks)		Term placenta (40 weeks)	
Control	0.48	3.92	0.48	20.22
L-Phenylalanine (5)	0.10	0.77	0.22	10.00
L-Tryptophan (5)	0.04	0.34	0.11	5.26
L-Leucine (5)	0.18	1.47	0.28	12.90

[a] μ mol *p*-nitrophenol liberated/mg protein/h.

Table 4 shows the thermostability (at 65°C for 10 min) of the enzymes. Alkaline phosphatase in early gestation was heat-labile. However, the enzyme was almost completely heat-stable as the pregnancy advanced. In contrast, the 5′-nucleotidase was stable during early period but the stability decreased during placental maturity.

Table 4. Effect of heat-treatment on alkaline phosphatase and 5′-nucleotidase from developing human placenta.

Gestation (in weeks)	Residual activity (%)	
	Alkaline phosphatase	5′-Nucleotidase
Early (8-10)	10	84
Mid (20-24)	99	64
Term (40)	99	47

For the study of heat-stability, the enzyme preparation was incubated at 65°C for 10 min, immediately cooled in ice, assayed for the remaining enzyme activity and compared with the control. The activity of the control was normalized to 100 and the residual activity in each case was expressed as per cent of this control value.

The results are the mean of 3 observations for each gestation.

The hydrolysis of *p*-nitrophenyl phosphate by alkaline phosphatase from the early placenta was activated by Ca^{2+}, Mg^{2+} and Mn^{2+}, but was strongly inhibited by Hg^{2+} and Zn^{2+}, while Cu^{2+}, Ni^{2+} and Co^{2+} caused only slight inhibition (table 5). The enzyme in placenta obtained at term was more resistant and was inhibited only by Hg^{2+} and Zn^{2+}. On the other hand, the metal ions tested did not

Table 5. The Effect of metal ions and EDTA on 5′-nucleotidase and alkaline phosphatase from human placenta

Metal ion added	Alkaline phosphatase		5′-Nucleotidase	
	Early placenta	Term placenta	Early placenta	Term placenta
None	100	100	100	100
Hg^{2+}	0	15	2	12
Ca^{2+}	136	116	95	98
Mg^{2+}	116	112	101	106
Mn^{2+}	120	112	41	97
Co^{2+}	91	99	98	100
Zn^{2+}	0	17	7	25
Cu^{2+}	76	93	81	82
Ni^{2+}	80	111	79	100
EDTA (1 mM)	49	78	79	90

The activity in the absence of any added metal ion was normalized to 100 and the activity in the-presence of these metal ions is expressed as per cent of this normalized acitivity.

The incubation medium contained the respective metal ion as its chloride salt to make a final concentration of 1 mM.

The results are the mean of 3 observations in each case.

stimulate the 5′-nucleotidase activity. While Hg^{2+} , Zn^{2+} and Cu^{2+} inhibited the enzyme activity, Ni^{2+} and Mn^{2+} caused only marginal inhibition in early gestation, but not in term placenta. Ca^{2} , Mg^{2} and Co^{2} were without effect.

The extent of inhibition by EDTA of both the enzymes decreased as the age of the placenta increased.

Table 6 shows the effect of Concanavalin A on the enzyme activities. It is observed that Concanavalin A inhibited 5′-nucleotidase in early placenta to a greater extent

Table 6. Effect of Concanavalin A on 5′-nucleotidase and alkaline phosphatase from human placenta.

Concanavalin A added (in μg)	Inhibition (%)			
	5′-Nucleotidase		Alkaline phosphatase	
	Early placenta	Term placenta	Early placenta	Term placenta
10	2	0	2	2
50	5	0	6	5
100	20	11	14	7
200	35	21	16	6
300	32	20	16	6

The results are the mean of 3 observations in each case.

than that in term placenta. The alkaline phosphatase behaved similarly, although the inhibition by the lectin was to a lesser extent.

Discussion

The much higher activities of alkaline phosphatase and 5′-nucleotidase activities in the microsomal fraction compared to that in cytosol indicated that these enzymes were mostly membrane-bound. The steady increase in the enzyme activities in early gestation was a characteristic of tissues in active proliferation and the decreased activities at term possibly reflected the decreased physiological function and ageing of the tissue. Similar observations have also been reported with ∝-glucosidase (Thanavala *et al*., 1974), acid phosphatase (Kushari *et al*., 1978), acetyl cholinesterase (Sastry *et al*., 1976) and ATPase (Chakraborti and Mukherjea, 1980).

The inhibition of alkaline phosphatase by L-phenylalanine, L-tyrosine and L-leucine is consistent with the findings of several investigators (Ghosh and Fishman, 1966; Lin *et al*., 1971; Nakayama *et al*., 1970). The reason for the progressively decreased inhibition of alkaline phosphatase by amino acids with increase in gestation is still not clear. This observation, however, strengthens the suggestion that this enzyme is metabolically modified during development (Fishman *et al*., 1976).

The measurement of 5′-nucleotidase activity can be affected by the presence of non-specific phosphatases. In this study, 5′-nucleotidase was assayed after inhibition of alkaline phosphatase with L-tryptophan. Complete inhibition of alkaline phosphatase was achieved at a concentration of 15 mM L-trytophan. In our study, a concentration of 20 mM L-tryptophan was routinely used to inhibit alkaline phosphatase.

The change in V_{max} of both the enzymes without affecting K_m indicated quantitative alteration of the enzymes during intra-uterine development of the placenta.

The physiological significance of the heat stability of alkaline phosphatase and 5′-nucleotidase in this rapidly developing tissue is still obscure. However, it is assumed that these types of enzymes possess higher half-life in the cells to support rapid growth and proliferation of the tissue (Neale *et al*., 1965).

The activation of alkaline phosphatase by Mg^{2+} and inhibition by Zn^{2+} in human placenta support the findings of Sugiura *et al*. (1977). Contradictory reports are available regarding the effects of EDTA on placental alkaline phosphatase. The present study is in agreement with that of placenta-like alkaline phosphatases from human osteosarcoma cells (Singh *et al*., 1978), but in contradiction with other reports (Kitchener *et al*., 1965; Conyers *et al*., 1966). That Mg^{2+} is almost ineffective on the activity of placental 5′-nucleotidase is consistent with the findings of Krishnakantha and Maguir (1978) but not with that of Fox and Pamela (1976). Inhibition of 5′-nucleotidase by Ni^{2+} (in early placenta), Zn^{2+} and Cu^{2+} is in conformity with the studies of 5′-nucleotidase from other sources (Ahmed and Reis, 1958).

The inhibition study of placental 5′-nucleotidase by Concanavalin A indicated the glycoprotein nature of the enzyme which is also supported by the studies with the placental enzyme (Krishnakantha and Maguir, 1978) as well as enzymes from other sources (Bhavadasan and Ganguly, 1976; Riordan and Slavik, 1974). Low inhibition of alkaline phosphatase activity by the lectin indicates that the carbohydrate content of the enzyme is very meagre. According to Ahmed and King (1969), human placental alkaline phosphatase contains only traces of carbohydrate, of which the major components are glucosamine and mannose (Hiroshi and Akagi, 1977).

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