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Purification and Properties of Human Alkaline Phosphatase from Meconium[1)]

Mamoru Sugiura,*,[a] Kazuyuki Hirano,[a] Yuichi Iizumi,[a] Jun Miyazaki,[b] Kazumasa Miki,[b] Shiro Iino,[b] Hiroshi Suzuki,[b] and Toshitsugu Oda[b]

Gifu College of Pharmacy,[a] 6-1, Mitahora-higashi 5 chome, Gifu 502, Japan and First Department of Internal Medicine, Faculty of Medicine, University of Tokyo,[b] 3-1, Hongo 7 chome, Bunkyo-ku, Tokyo 113, Japan

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Human alkaline phosphatase was purified from meconium by treatment with cetylpyridinium chloride, followed by diethylaminoethyl (DEAE)-cellulose and CM-cellulose chromatography, Sephadex G-200 gel filtration and DEAE-Sephadex A-50 chromatography. The homogeneity of the purified enzyme was demonstrated by disc electrophoresis and immunological investigation. The molecular weight of the purified meconial alkaline phosphatase was 155000 and the enzyme was composed of two subunits of equal molecular weight. The optimum pH was found to be 10.0 and the enzyme was stable over the pH range of 4—10. The *K*m value was 2.2 mM for *p*-nitrophenylphosphate as a substrate. The isoelectric point was pH 4.0, and the purified enzyme was inhibited by N-bromosuccinimide (NBS), *o*-phenanthroline, ethylenediaminetetraacetic acid (EDTA) and L-phenylalanine. Meconial alkaline phosphatase obtained by our method contained 2 g-atoms of zinc/mole of enzyme. The enzymic properties of the purified meconial alkaline phosphatase were compared with those of adult intestinal alkaline phosphatase.

Keywords——meconial alkaline phosphatase; adult intestinal alkaline phosphatase; purification; properties; sialic acid

Introduction

Human alkaline phosphatase (EC 3.1.3.1) exists in various organs in multiple forms having distinct properties.[2,3)] These tissue-specific alkaline phosphatases originate from the liver, bone, bile, intestine and placenta. As part of a series of studies on human alkaline phosphatase, we have previously reported the purification and some properties of human alkaline phosphatases from placenta,[4)] intestine,[5)] liver,[6)] kidney[7)] and bile.[8)] The electrophoretic mobility of human fetal intestinal alkaline phosphatase was different from that of adult intestinal alkaline phosphatase.[9)] The enzymic properties of fetal intestinal alkaline phosphatase were quite similar to those of hepatoma alkaline phosphatase.[10)] However, the details of these properties have not been described. In the fetal tissues the highest activity of alkaline phosphatase exists in meconium and neonatal feces. Therefore, alkaline phosphatase from meconium and neonatal feces may originate from the fetal and neonatal intestine, and this should be reflected in the enzymic and immunological properties. This paper deals with the purification and some properties of meconial alkaline phosphatase and with a comparison of the enzymic properties of meconial and adult intestinal alkaline phosphatases.

Materials and Methods

Materials——DEAE-cellulose and carboxymethyl (CM)-cellulose were obtained from Brown Co., Ltd. Sephadex G-200 and DEAE-Sephadex A-50 were purchased from Pharmacia Fine Chemicals. Neuraminidase was obtained from Boehringer Mannheim Co., Ltd. The other reagents were of analytical reagent grade.

Preparation of Crude Enzyme——Meconium (50 g) was homogenized with distilled water and *n*-butanol (meconium: water: *n*-butanol = 1: 3: 1). The supernatant was used as the crude enzyme solution.

Assay of Alkaline Phosphatase Activity——Method A: Disodium phenylphosphate was used as a substrate.[11)] A 2 ml aliquot of 0.1 M carbonate buffer (pH 10.5) containing 10 mM substrate and 2 mM 4-

aminoantipyrine was preincubated at 37°C for 5 min. The enzyme solution was added and the enzyme reaction was carried out at 37°C for 15 min. The reaction was stopped by the addition of 2 ml of 0.2 M boric acid solution containing 6 mM potassium ferricyanide, and the absorbance of the resulting solution was determined at 500 nm. One unit of alkaline phosphatase activity was defined as the amount which liberated 1 μmol of phenol per min at 37°C.

Method B: *p*-Nitrophenylphosphate was used as a substrate.[12] One ml of 10 mM substrate solution and 3 ml of 0.1 M glycine–KCl– KOH buffer (pH 10.5) were preincubated at 37°C for 5 min. One ml of the enzyme solution was added and the enzyme reaction was carried out at 37°C for 30 min. The reaction was stopped by adding 2 ml of 0.1 N NaOH. The extent of hydrolysis was determined from the absorbance of *p*-nitrophenol at 405 nm. Method B was used for the study of the properties of meconial alkaline phosphatase.

Determination of Protein——Protein content was determined either by measuring the absorbance at 280 nm or by the method of Lowry *et al.*[13] with bovine serum albumin as a standard.

Polyacrylamide Gel Electrophoresis——Polyacrylamide gel electrophoresis under non-denaturing conditions was based on the procedure of Davis *et al.*[14] Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed by the method of Weber and Osborn.[15] The gels were stained for protein with Amido Black 10B. Alkaline phosphatase activity was detected by the simultaneous capture method.[16] The gels were incubated at 25°C for several minutes in 50 mM carbonate buffer (pH 10.2) containing 1 mg/ml α-naphthyl phosphate and 0.1 mg/ml of Fast Blue BB salt.

Determination of Molecular Weight——The molecular weight of native meconial alkaline phosphatase was determined by Sephadex G-200 gel filtration using cytochrome C, bovine serum albumin, γ-globulin and catalase as standards. The Sephadex G-200 column (2 × 70 cm) was equilibrated and eluted with 10 mM Tris–HCl buffer (pH 8.0) containing 0.1 M NaCl. The molecular weight of the purified enzyme subunit was determined by SDS polyacrylamide gel electrophoresis using ovalbumin, bovine serum albumin, phosphorylase a and β-galactosidase as standards. SDS-polyacrylamide gel electrophoresis was performed on 5% polyacrylamide gel containing 0.1% SDS in 0.1 M sodium phosphate buffer (pH 7.2), and protein bands were stained with Amido Black 10B.

Neuraminidase Treatment——One mg of neuraminidase (0.6 U) was dissolved in one ml of 0.1 M acetate buffer (pH 5.5) containing 0.15 M NaCl and 10 mM $MgCl_2$. The enzyme preparation was incubated with an equal volume of neuraminidase solution at 37°C for 20 h.

Isoelectrofocusing——Isoelectrofocusing was performed by the method of Vesterberg and Svensson.[17]

Determination of Sugars by Gas-Liquid Chromatography——The purified meconial alkaline phosphatase preparation to be analyzed for sugars was extensively dialyzed against distilled water and lyophilized. The conditions for hydrolysis, preparation of trimethylsilyl derivatives and their analysis by gas-liquid chromatography were principally those of Sweeley and Walker.[18] A column (2 ml) of 3% OV-1 on Gas Chrom Z was employed. Analysis was performed with a Shimadzu model 4BM-PF gas-liquid chromatography, which was equipped with a hydrogen flame detector. Derivatives were identified from their retention times relative to the internal standard (mannitol). The operating conditions were: oven temperature, 165°C; detector temperature, 300°C; nitrogen flow-rate, 40 ml/min; air flow-rate, 700 ml/min; and hydrogen flow-rate, 55 ml/min. The column temperature was raised at 4°C/min.

Amino Acid and Amino Sugar Analyses——The amino acid and amino sugar analyses were performed according to the method of Liu and Chang[19] with a JEOL amino acid autoanalyzer, model JLC-6AH. Protein samples for amino acid and amino sugar analyses were hydrolyzed with 3 N *p*-toluenesulfonic acid containing tryptamine at 110°C for 48 h in evacuated sealed tubes.

Determination of Metal Content of Meconial Alkaline Phosphatase——The purified enzyme was dialyzed against distilled water for 48 h then lyophilized, weighed and dissolved in 10 ml of 1 N HCl. Metal content was determined with a Shimadzu AA-610S atomic absorption spectrophotometer.

Preparation of Antibody against Meconial Alkaline Phosphatase——A female rabbit was inoculated subcutaneously with 1 mg of the purified enzyme emulsified in complete Freund's adjuvant. Two and four weeks later, booster injections of 1 mg of the purified enzyme emulsified in complete Freund's adjuvant were given. After a somewhat longer interval another booster injection was given as above. Two weeks later, the rabbit was bled and the immunoglobulin G fraction was purified.

Results

Purification of Meconial Alkaline Phosphatase

All procedures were carried out at 4°C. In order to remove pigment in the meconium, cetylpyridinium chloride (CPC) was added to the crude enzyme solution. The pigment was almost completely precipitated at CPC concentrations of above 0.2% (W/W), as shown in Fig. 1. The CPC-treated enzyme solution was exclusively dialyzed against 10 mM Tris–HCl buffer (pH 7.4) containing 10 μM $MgCl_2$ and $ZnCl_2$, and applied to a DEAE-cellulose column

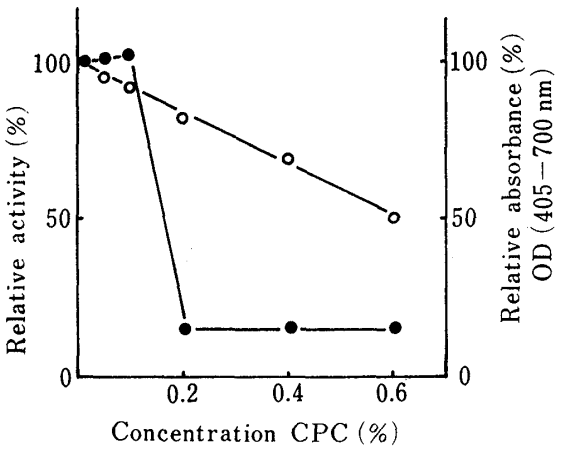

Fig. 1. Removal of the Pigment in Meconial Alkaline Phosphatase

CPC was added to the stirred crude enzyme solution, and the activity and the absorbance value at 405 nm and 700 nm were measured.
—○—: relative activity.
—●—: relative absorbance.

(3×40 cm) which had been equilibrated with the same buffer. The column was washed with the above buffer, and the enzyme was eluted with a linearly increasing sodium chloride gradient (0—0.3 M). The active fractions were concentrated with a UM-10 membrane filter and the solution was exchanged for 10 mM citrate buffer (pH 5.0) containing 10 μM $MgCl_2$ and $ZnCl_2$. The enzyme was passed through a column of CM-cellulose (3×25 cm), which had been equilibrated with the above buffer. The active fractions were dialyzed against 10 mM Tris–HCl buffer (pH 8.0) containing 0.1 M NaCl, 10 μM $MgCl_2$ and $ZnCl_2$. The dialyzed enzyme was concentrated with a UM-10 membrane filter and applied to a column of Sephadex G-200 (3.5×90 cm) which had been equilibrated with the same buffer. The active fractions were collected and dialyzed against 20 mM Tris–HCl buffer (pH 7.4) containing 10 μM $MgCl_2$ and $ZnCl_2$. The dialyzed enzyme was absorbed on a DEAE–Sephadex A-50 column (3.5×30 cm) which had been equilibrated with the same buffer. The column was washed with the same buffer, and the absorbed enzyme was eluted with a linear gradient of 0 to 0.3 M sodium chloride. The elution profiles of these column chromatography procedures are shown in Fig. 2. The active fraction were collected and dialyzed against distilled water. The above purification procedures are summarized in Table I. Meconial alkaline phosphatase was purified approximately 330-fold from the crude enzyme with a recovery of 3.8%. The homogeneity of the purified enzyme was confirmed by disc electrophoresis and SDS polyacrylamide gel electrophoresis as shown in Fig. 3.

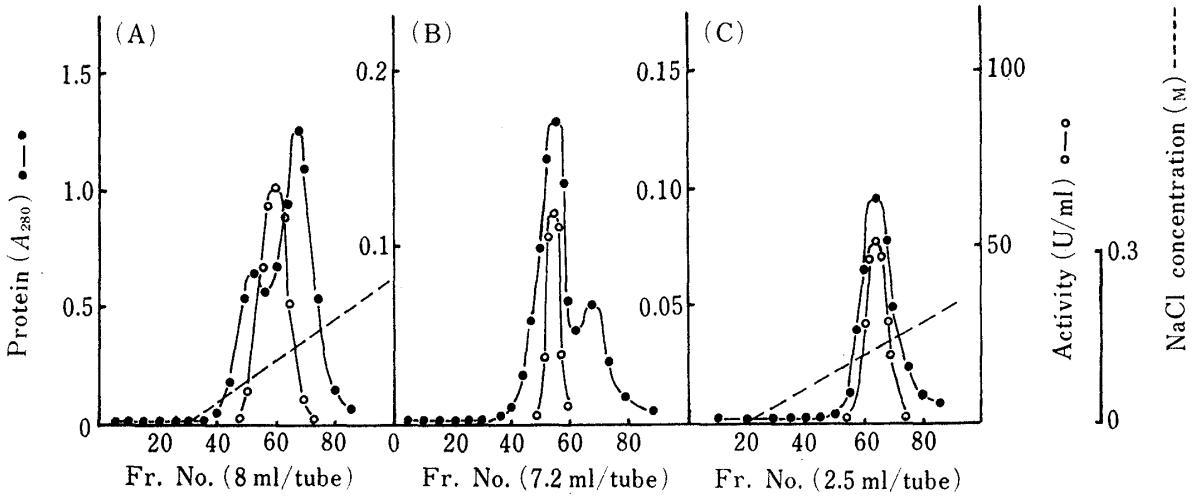

Fig. 2. Column Chromatographies of Alkaline Phosphatase from Meconium on DEAE-cellulose (A), Sephadex G-200 (B) and DEAE-Sephadex A-50 (C)

(A): After the treatment with CPC, the crude enzyme was applied to a DEAE-cellulose column (3×40 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.4) containing 10 μM $MgCl_2$ and $ZnCl_2$. The enzyme was eluted with a linear NaCl gradient from 0 to 0.3 M. (B): The active fractions from CM-cellulose were applied to a Sephadex G-200 column (3.5×90 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 10 μM $MgCl_2$, $ZnCl_2$ and 0.1 M NaCl. (C): The active fractions from Sephadex G-200 were applied to a DEAE-Sephadex A-50 column (3.5×30 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.4) containing 10 μM $MgCl_2$ and $ZnCl_2$. The enzyme was eluted with a linear NaCl gradient from 0 to 0.3 M.

Table I. Purification of Alkaline Phosphatase from Meconium

Step of purification	T.A. (units)	T.P. (mg)	S.A. (units/mg)
Crude	39400	18600	2.12
CPC treatment	31800	11200	2.84
DEAE-cellulose column chromatography	5410	62.3	86.9
CM-cellulose column chromatography	3580	7.9	453
Sephadex G-200 column chromatography	2490	4.0	623
DEAE-Sephadex A-50 column chromatography	1490	2.1	708

The activity was measured by the Kind-King method and protein was measured by the method of Lowry.
T.A.: total activity. T.P.: total protein. S.A.: specific activity.

Enzymic Properties of Meconial Alkaline Phosphatase Effect of pH on Activity and Stability

The optimum pH of this enzyme was investigated with Britton-Robinson buffers having various pH values. The optimum pH was found to be 10.0. For the purpose of examining its pH stability, the enzyme was incubated with the buffers having various pH values at 37°C for 30 min and found to be stable in the pH range of 4—10.

Molecular Weight

Gel filtration on Sephadex G-200 suggested that the molecular weight of native meconial alkaline phosphatase was 155000, and SDS polyacrylamide gel suggested that the molecular weight of the enzyme subunit was 75000. These results reveal that meconial alkaline phosphatase consists of two subunits, which have identical molecular weight.

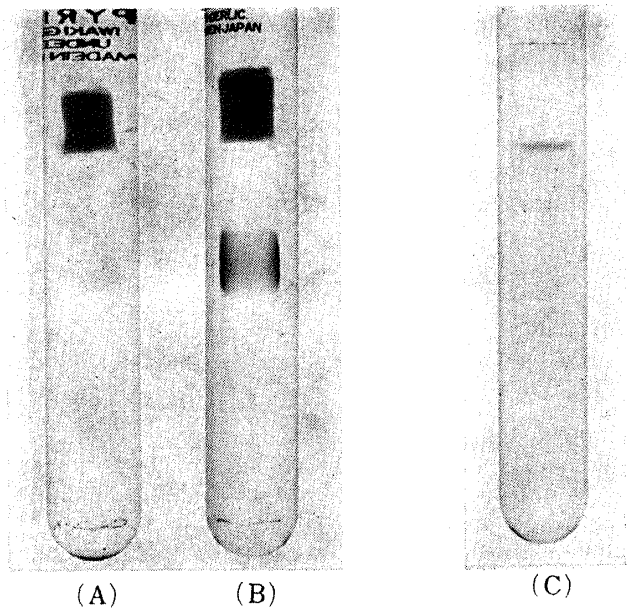

Fig. 3. Polyacrylamide Disc Electrophoresis and SDS Polyacrylamide Gel Electrophoresis of Alkaline Phosphatase from Meconium

Disc electrophoresis was performed on 7.5% polyacrylamide gel. The gels were stained for enzyme activity (A) and for protein (B) as described in the text. SDS polyacrylamide gel electrophoresis was performed on 5% polyacrylamide gel (C).

Michaelis Constant

The Michaelis constant of meconial alkaline phosphatase was 2.2 mM with *p*-nitrophenylphosphate as a substrate. This value was not influenced by treatment with neuraminidase.

Effect of Various Metal Salts and Reagents on the Activity

The meconial alkaline phosphatase activity was measured in the presence of metal salts or reagents. The results are shown in Table II. The purified enzyme was inhibited by NBS and chelators (*o*-phenanthroline and EDTA), but it was slightly activated by $ZnCl_2$ and $MnCl_2$. These results indicated that the meconial alkaline phosphatase is a metalloenzyme. In the presence of L-cysteine, the enzyme activity was strongly inhibited, and this enzyme was slightly

Table II. Effects of Various Metal Salts and Reagents on the Activity of Alkaline Phosphatase from Meconium

Metal salt[a] or reagent	Remaining activity (%)	Amino acid[b]	Remaining activity (%)
None	100	L-Cysteine	0
$MnCl_2$	124	L-Phenylalanine	72
$ZnCl_2$	113	L-Tryptophan	65
N-Bromosuccinimide	0		
o-Phenanthroline	12		
EDTA	13		

a) The enzyme was incubated with 0.1 mM metal salt or 1 mM reagent in 50 mM Tris-HCl buffer (pH 7.4) at 37°C for 30 min. The incubation mixture was diluted 100-fold with 10 mM Tris-HCl buffer (pH 7.4), and the remaining activity was measured.
b) The enzyme activity was determined in the presence of various amino acids (1 mM).

inhibited in the presence of L-phenylalanine and L-tryptophan. The enzyme activity was not influenced by various other compounds.

Isoelectric Point of Meconial Alkaline Phosphatase

The isoelectric point of the purified enzyme was investigated by the isoelectrofocusing technique. The purified enzyme appeared as a single band at around pH 4.0.

Sugar Contents of Meconial Alkaline Phosphatase

Sugar contents of the purified enzyme were determined. As shown in Table III, mannose, galactose, glucose, fucose and sialic acid were detected in the purified enzyme.

Table III. Sugar Composition of Alkaline Phosphatase from Meconium

Sugar	Content[a]
Fucose	0.111
Mannose	0.018
Galactose	0.266
Glucose	0.101
Sialic acid	0.041

a) Sugar contents of this enzyme are expressed as mg per mg of protein. Other conditions were as described in the text.

Amino Acid and Amino Sugar Compositions of Meconial Alkaline Phosphatase

Amino acid and amino sugar compositions of the purified enzyme are shown in Table IV. Compared with the adult intestinal alkaline phosphatase, acidic amino acids accounted for a larger part of the total amino acid composition of the purified enzyme, and this result was consistent with the isoelectric point of this enzyme, pH 4.0. The amino sugar content of this enzyme was also investigated, and was larger than that of adult intestinal alkaline phosphatase.

Zinc Content in Meconial Alkaline Phosphatase

Zinc content in the purified enzyme obtained by our method was determined with an atomic absorption spectrophotometer. The purified enzyme contained 2 g-atoms of zinc/mole of enzyme.

Immunological Study of Meconial Alkaline Phosphatase

The purified enzyme was assessed by Ouchterlony immunodiffusion analysis. As can be seen from Fig. 4, the purified meconial alkaline phosphatase showed only a single precipitation line against anti-meconial alkaline phosphatase antibody. However, the meconial

Table IV. Amino Acid and Amino Sugar Compositions of Alkaline Phosphatase from Meconium

Amino acid and amino sugar	Residues
Trp	16
Lys	31
His	19
Arg	28
Asp	95
Thr	133
Ser	101
Glu	91
Pro	195
Gly	98
Ala	98
1/2 Cys	3
Val	82
Met	12
Ile	22
Leu	57
Tyr	22
Phe	22
Glucosamine	174
Galactosamine	73

The protein was hydrolyzed with 3 N *p*-toluenesulfonic acid at 110°C for 48 h. The number of residues is expressed as residues/mole of enzyme protein.

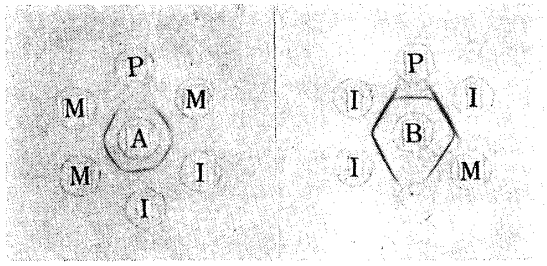

Fig. 4. Immunodoublediffusion of Alkaline Phosphatases

A: anti-meconial alkaline phosphatase antibody.
B: anti-placental alkaline phosphatase antibody.
M: purified meconial alkaline phosphatase.
I: purified adult intestinal alkaline phosphatase.
P: purified placental alkaline phosphatase.

enzyme was precipitated against anti-adult intestinal alkaline phosphatase antibody. Placental alkaline phosphatase was precipitated against antibodies to adult intestinal and meconial enzymes. Thus, placental, adult intestinal and meconial alkaline phosphatases share partially cross-reactive antigenic determinants.

Discussion

Meconial alkaline phosphatase was extracted from meconium by treatment with cetylpyridinium chloride and purified by DEAE-cellulose and CM-cellulose chromatography, Sephadex G-200 gel filtration and DEAE-Sephadex A-50 chromatography. The alkaline phosphatase activity coincided with the protein band on polyacrylamide gel electrophoresis of the purified meconial enzyme, which also showed only a single precipitation line against anti-meconial alkaline phosphatase antibody in Ouchterlony immunodiffusion. Thus, the homogeneity of the purified enzyme was demonstrated by disc electrophoresis, SDS polyacrylamide electrophoresis and immunological investigations. The enzymic properties of the purified enzyme were compared with those of adult intestinal alkaline phosphatase.[5,20] The molecular weight determined by gel filtration was 155000 for meconial alkaline phosphatase, while that of adult intestinal alkaline phosphatase was 170000. Moreover, the molecular weights of meconial and adult intestinal enzyme subunits determined by SDS polyacrylamide gel electrophoresis were 75000 and 86000, respectively. The above results suggest that meconial alkaline phosphatase is composed of two subunits of equal or very similar molecular weight. The pH stability and optimum pH of meconial alkaline phosphatase were similar to those of adult intestinal alkaline phosphatase. The heat stabilities of these two enzymes were similar. The inhibitions by L-phenylalanine and EDTA of meconial and adult intestinal alkaline phosphatases were quite similar. $HgCl_2$ and $CdCl_2$ did not inhibit the meconial enzyme, while the adult intestinal alkaline enzyme was strongly inhibited. However, the effects of various other compounds and reagents on these two enzymes were similar. Generally, meconial alkaline phosphatase has low sensitivity to these compounds and reagents. The isoelectric point of this enzyme was at pH 4.0, and this value is low in comparison with that of adult

intestinal alkaline phosphatase. In comparison with adult intestinal alkaline phosphatase, acidic amino acids were found to account for a larger part of the total amino acid composition of the purified enzyme. Sialic acid was found in the meconial enzyme, but was not detected in the adult intestinal enzyme. The electrophoretic mobility of meconial alkaline phosphatase was different from that of adult intestinal alkaline phosphatase, and the former had a broad band of activity on polyacrylamide gel compared with the latter, but on treatment with neuraminidase, the broad band of activity of meconial alkaline phosphatase narrowed and its mobility coincided with that of adult intestinal alkaline phosphatase. These observations suggest that the difference of mobility between meconial and adult intestinal alkaline phosphatases is due their sialic acid content. The amino sugar content of meconial alkaline phosphatase was larger than that of adult intestinal alkaline phosphatase. The purified enzyme obtained by our method contained 2 g-atoms of zinc/mole of enzyme, while adult intestinal alkaline phosphatase contains 4 g-atoms of zinc/mole of enzyme. As a whole, the enzymic properties of meconial alkaline phosphatase are similar to those of adult intestinal alkaline phosphatase. Meconial enzyme was precipitated by antibodies to meconial and adult intestinal alkaline phosphatases. This finding suggests that meconial and adult intestinal enzymes at least partially share cross-reactive antigenic determinants. On the basis of these results, it is suggested that meconial and adult intestinal alkaline phosphatase may be coded by the same gene.

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