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Characterization of Human Tissue-specific Alkaline Phosphatase[1)]

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Among various tissue-specific alkaline phosphatases, sialic acid was detected in liver, placental and meconial alkaline phosphatases, but not in the intestinal one. The content of acidic amino acids was larger than that of basic amino acids in the purified enzymes. Placental, intestinal and liver alkaline phosphatases contained 4 g-atoms of zinc/mol of enzyme, but the meconial alkaline phosphatase contained 2 g-atoms of zinc/mol of enzyme. N-Terminal amino acid residues of the intestinal and meconical alkaline phosphatases were both phenylalanine, whereas that of placental enzyme was isoleucine and that of liver enzyme was leucine. The tryptic peptide patterns of human placental, intestinal, meconial and liver alkaline phosphatases were similar to one another in part. The active-center-containing peptides labelled with $^{32}PO_4$ from human placental, intestinal, meconial and liver alkaline phosphatases had the same mobility on a thin layer chromatogram.

Keywords——human tissue-specific alkaline phosphatase; enzymic property; immunological property; structural relationships; active-center-containing peptide

Introduction

The level of serum alkaline phosphatase (EC 3.1.3.1) is a very important parameter in clinical diagnosis. This enzyme exists in various tissues in multiple forms having distinct properties.[2,3)] These tissue-specific alkaline phosphatase originate from liver, bone, intestine and placenta. For this reason, it is necessary to understand the structural relationship of human tissue-specific alkaline phosphatases. The function and structure of *E. coli* alkaline phosphatase have been well studied by Schesinger and Barrett.[4)] However, few structural relationships are known concerning alkaline phosphatase from mammalian cells, especially from human tissues. 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 liver, placenta, intestine and meconium.[5–8)] In this work, these tissue-specific alkaline phosphatases were compared with one another as regards enzymic and immunological properties and structural relationship (sugar composition, amino acid composition, zinc content, N-terminal amino acid residues and tryptic peptide patterns). The active-center-containing peptides labelled with $^{32}PO_4$ were compared with one another by autoradiography.

Materials and Methods

Materials——Cellulose powder (C type) for the peptide mapping plates was obtained from Toyo Roshi Co., Ltd. Polyamide was purchased from Seikagaku Kogyo Co., Ltd., Carrier-free[^{32}P] orthophosphate was from New England Nuclear Corp. All other reagents were of analytical grade quality.

Enzymes——The purifications of human alkaline phosphatase from liver, placenta, intestine and meconium were performed as previously described.[5–8)] The homogeneity of the purified enzymes were demonstrated by disc electrophoresis and immunological investigation. TPCK-trypsin was obtained from Worthington Biochemical Corp.

Assay of Alkaline Phosphatase Activity——Alkaline phosphatase activity was measured in all experiments by the method of Bessey *et al.*[9)] in 0.1 M glycine–KCl–KOH buffer (pH 10.5) with *p*-nitrophenylphosphate as a substrate.

Determination of Sugar Composition——A purified enzyme preparation to be analyzed for sugar composition 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.[10] A column (2 ml) of 3% OV-1 on Gas Chrom Z was employed. Analysis was performed with a Shimadzu 4BM-PF gas–liquid chromatograph, which was equipped with a hydrogen flame detector. Derivatives were identified from their retention times relative to that of 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; hydrogen flow-rate, 55 ml/min. The column temperatures was raised at 4°C/min.

Amino Acid and Amino Sugar Analyses——Enzymes were hydrolyzed with 3 N *p*-toluenesulfonic acid containing tryptamine at 110°C for 48 h in evacuated sealed tubes, and amino acid and amino sugar were analyzed according to the method of Liu and Chang[11] with a JEOL amino acid autoanalyzer, model JLC-6AH.

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

Analysis of *N*-Terminal Amino Acid Residue——The N-terminal amino acid residue analysis was performed by the method of Woods and Gray[12,13] and the method of Weiner *et al.*[14] Protein was dissolved at 100 μg per 50 μl of 1% sodium dodecyl sulfate (SDS) solution by heating with boiling water. Then 50 μl of *N*-ethylmorpholine and 75 μl of dansyl chloride (2.5% w/v, dimethylformamide anhydrous) were added to this solution. After dansylation for 1 h at room temperature, the dansylated protein was precipitated by adding 0.5 ml of acetone. After centrifugation the supernatant was withdrawn, and 50 μl of 6 N HCl was added. After hydrolysis for 18 h at 105°C, the hydrolysates were dried in a vacuum desiccator. The dansyl-amino acid was dissolved in 10 μl of 50% aqueous pyridine (v/v) and was spotted onto one side of a polyamide thin layer. Solvent I was run in the first dimension, and solvent II was run perpendicular to solvent I.

Solvent I: 1.5% formic acid in water.

Solvent II: benzene–acetic acid, 9: 1.

The chromatogram was visualized under ultraviolet light.

Peptide Map Pattern——The analysis of peptide map patterns was performed by the method of Kanarek *et al.*[15] The tryptic digests of human alkaline phosphatase were prepared under the following conditions. The reaction mixture (13 ml) contained 1.83 mg of lyophilized enzyme, 0.5 M Tris–HCl buffer (pH 8.6) containing 0.2% ethylenediaminetetraacetic acid (EDTA), 8 M urea and 0.04 M 2-mercaptoethanol. After 30 min, the mixture was made up to 0.1 M iodoacetic acid by the addition of the solid acid. The mixture was maintained at pH 8.6 by the addition of 2.5 N NaOH. The reaction appeared to be complete within 5 min as judged by the absence of further change in pH. The mixture was allowed to react for an additional 15 min. 2-Mercaptoethanol, in an amount sufficient to give a final concentration of 0.15 M, was added, and the pH was maintained at 8.6 by the addition of 2.5 N NaOH. After standing for 5 min, the mixture was dialyzed exhaustively against distilled water. The final product was recovered by lyophilization. For tryptic digestion, 1.1 mg of the lyophilized enzyme was dissolved in 0.2 ml of dilute NH_4OH and adjusted to pH 8.5 with 0.2 M NH_4HCO_3. The solution was incubated at 40°C. Eleven μg of TPCK-trypsin dissolved in 50 μl of 0.001 N HCl was added. After 1 h, a further 11 μg of TPCK-trypsin was added, and the digestion allowed to proceed overnight. The final product was lyophilized and dissolved in 160 μl of 4 M NH_4OH. Peptide maps of the digests were made under the following conditions. A sample of the tryptic peptides was applied to a cellulose-coated thin layer plate (20 × 20 cm). Electrophoresis was run first, followed by chromatography. Electrophoresis was performed in acetic acid/formic acid/water (15: 5: 80, by vol.) at 500 V for 60 min. After the plate had been dried at 70°C for 15 min, second dimensional chromatography was run for 90 min with *n*-butanol/pyridine/acetic acid/water (65: 50: 10: 40, by vol.) as a solvent. Peptides were stained with fluorescamine.

Analysis of Active-center-containing Peptide labelled with $^{32}PO_4$——Placental, intestinal, meconial and liver alkaline phosphatases were labelled with [^{32}P] orthophosphate under the following conditions.[16] The reaction mixture (5 ml) consisted of 1 mg of each enzyme, 50 mM acetate buffer (pH 5.0), 20 mM disodium hydrogen phosphate and 1 mCi carrier-free [^{32}P] orthophosphate. After 5 min at 0°C, perchloric acid was added to a final concentration of 0.67 M to precipitate the enzyme. The precipitate was washed several times with 0.5 M perchloric acid to remove unbound phosphate. The labelled alkaline phosphatase precipitate was suspended in 3.3 ml of 0.5 M ammonium carbonate (pH 8.5). One hundred μg of TPCK-trypsin dissolved in 100 μl of 0.5 M ammonium carbonate was added and the mixture was incubated at 37°C for 24 h. The tryptic peptide was lyophilized to remove ammonium carbonate. Electrophoresis and chromatography were performed on a cellulose-coated thin layer plate (20 × 20 cm). For plates run in two dimensions, electrophoresis was run first, followed by chromatography. Electrophoresis was performed in acetic acid/formic acid/water (15: 5: 80, by vol.) at 500 V for 40 min. After the plate had been dried at 70°C for 15 min, chromatography was run for 90 min with *n*-butanol/pyridine/acetic acid/water (65: 50: 10: 40, by vol.) as a solvent. Radioactivity of labelled peptide was detected by autoradiography.

Antibodies——Antibodies were obtained in rabbits immunized with the purified alkaline phosphatases in complete Freund's adjuvant according to the previous reports.[5-8]

Results and Discussion

Sugar Composition

The sugar compositions of the purified enzymes were determined. As shown in Table I, fucose, mannose, galactose and glucose were detected in the purified enzymes. Sialic acid was detected in placental, liver and meconial enzymes, but not in the intestinal enzyme. The sugar content of meconial alkaline phosphatase was larger than those of other alkaline phosphatases. In particular, meconial alkaline phosphatase had a broad band of activity on polyacrylamide gel compared with that of intestinal alkaline phosphatase. However, after treatment of the meconial enzyme with neuraminidase, the band of activity narrowed and its mobility became identical with that of the intestinal enzyme. This result suggests that the difference of mobility between meconial and intestinal alkaline phosphatases was due to a difference of sialic acid content.

Table I. Sugar Composition of Human Alkaline Phosphatases from Liver, Intestine, Meconium and Placenta

Sugar[a]	Liver	Intestine	Meconium	Placenta
Fucose	32	62	100	2
Mannose	85	63	16	13
Galactose	36	130	230	38
Glucose	3	1	87	1
Sialic acid	13	N.D[b]	31	28

a) The sugar content is expressed as mol/mol of enzyme protein.
b) Not detectable.

Table II. Amino Acid and Amino Sugar Compositions of Human Alkaline Phosphatases from Liver, Intestine, Meconium and Placenta

Residue	Liver	Intestine	Meconium	Placenta
Trp	9	9	16	8
Lys	83	58	31	60
His	45	34	19	38
Arg	67	71	28	73
Asp	151	187	95	95
Thr	115	100	133	77
Ser	103	95	101	62
Glu	149	150	91	120
Pro	92	100	195	68
Gly	117	100	98	100
Ala	144	107	98	117
1/2 Cys	11	5	3	8
Val	106	78	82	79
Met	38	26	12	33
Ile	52	60	22	40
Leu	131	122	57	95
Tyr	63	73	22	43
Phe	54	68	22	44
Glucosamine	68	44	174	14
Galactosamine	9	7	73	3

The number of residue is expressed as mol/mol of enzyme protein.

Amino Acid and Amino Sugar Compositions

The amino acid and amino sugar compositions of the purified enzymes are shown in Table II. The content of acidic amino acids was larger than that of basic amino acids in the enzymes. These results are consistent with the finding that the isoelectric points of these enzymes were low. The amino sugar compositions of these enzymes were also investigated in the same way. The amino sugar content of meconial alkaline phosphatase was larger than those of other alkaline phosphatases.

Zinc Content

The zinc contents in these enzymes were measured with an atomic absorption spectrophotometer. Placental, liver, intestinal and meconial alkaline phosphatases contained 4,4, 4 and 2 g-atoms of zinc/mol of enzyme, respectively.

N-Terminal Amino Acid Residue

The *N*-terminal amino acid residues of human alkaline phosphatases from liver, placenta, intestine and meconium were leucine, isoleucine, phenylalanine and phenylalanine, respectively.

Peptide Map Patterns

The peptide map patterns of human alkaline phosphatases from liver, placenta, intestine and meconium are shown in Fig. 1. The peptide map patterns, expressed as closed circles, of placental, intestinal and liver alkaline phosphatases were very similar. In particular, part of the patterns of placental and intestinal alkaline phosphatases were nearly identical. The

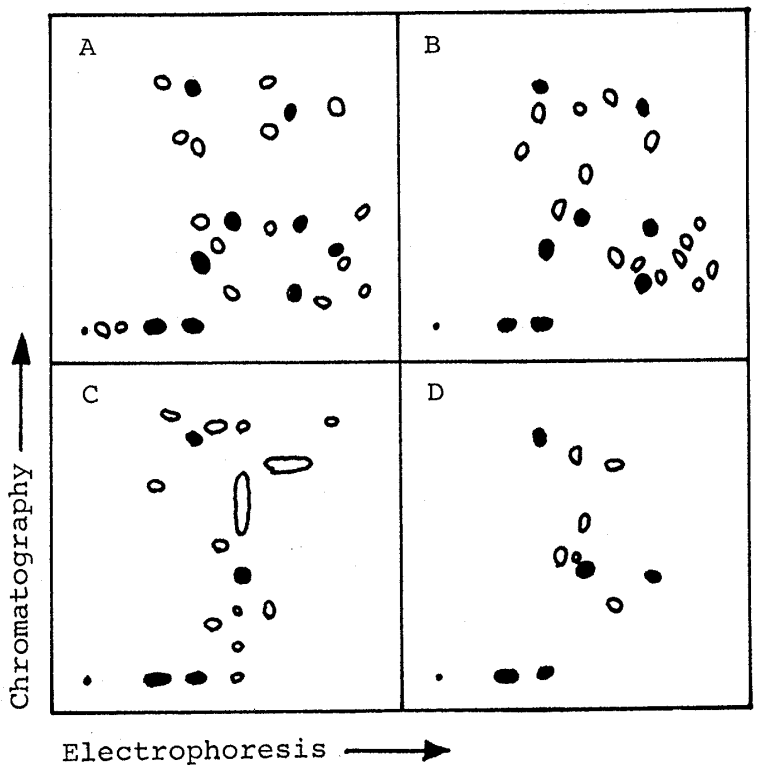

Fig. 1. Peptide Map Patterns of Human Alkaline Phosphatase from Placenta, Intestine, Liver and Meconium

A cellulose-coated TLC plate was moistened with acetic acid/formic acid/water (15 : 5 : 80) and electrophoresis was performed at 500 V, 4°C for 60 min. The plate was then chromatographed in a second direction with *n*-butanol/pyridine/acetic acid/water (65: 50: 10: 40), and the map was reproduced by tracing after detection with a fluorescamine spray. Enzyme from: A, placenta; B, intestine; C, liver; D, meconium.

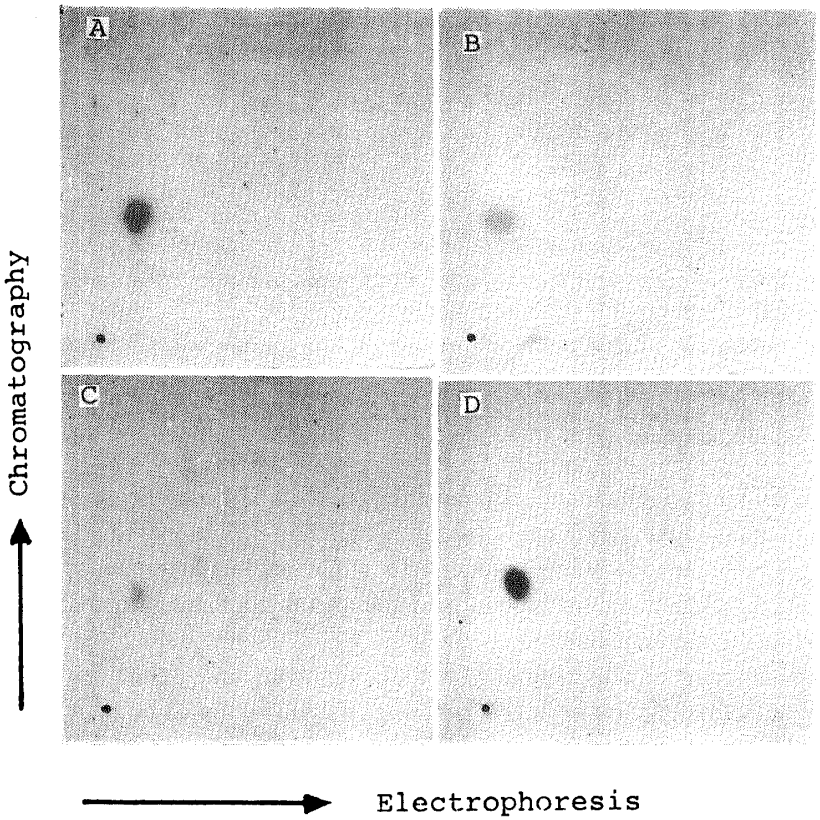

Fig. 2. Autoradiography of the Active-center-containing Peptides labelled with $^{32}PO_4$ from Human Alkaline Phosphatases of Liver, Placenta, Intestine and Meconium

The conditions are detailed in the text. The point of application was marked a closed circles. Enzyme from: A, liver; B, placenta; C, intestine; D, meconium.

patterns of meconial alkaline phosphatase was very similar to part of the pattern for intestinal alkaline phosphatase. These results suggest that the consistent peptides of meconial alkaline phosphatase were very similar to some of those of intestinal alkaline phosphatase.

Analysis of Active-center-containing Peptide labelled with $^{32}PO_4$

The tryptic peptide patterns of $^{32}PO_4$ labelled active-center-containing peptide from liver, placental, intestinal and meconial alkaline phosphatases are shown in Fig. 2. The free orthophosphate moved towards the anode because of its negative charge at pH 2. The radioactive spot moving towards the cathode represents phosphorylated peptide. The major radioactive spot of autoradiography was assumed to be the active-center-containing peptide. The mobilities of the radioactive spots from the enzymes were all very similar or the same. These results indicate that the active-center containing peptides of these four tissue-specific alkaline phosphatases are all very similar. Sussman and Stinson have reported that the ^{32}P- or ^{125}I-labelled denatured subunits of each enzyme were distinguished into three groups.[17,18] We investigated the structural relationships among the four enzymes in terms of the ^{32}P-labelled active-center-containing peptides. The results provide strong support for the view that at least the active-center-containing peptides of each enzyme are all identical. We are now determining the primary structures of the active-center-containing peptides of these enzymes.

Immunological Relationships of Human Tissue-specific Alkaline Phosphatases

Immunological relationships of human alkaline phosphatases from liver, placenta, intestine and meconium are shown in Fig. 3. Liver alkaline phosphatase was precipitated by anti-liver alkaline phosphatase antibody, but the liver enzyme did not react with antibodies to placental

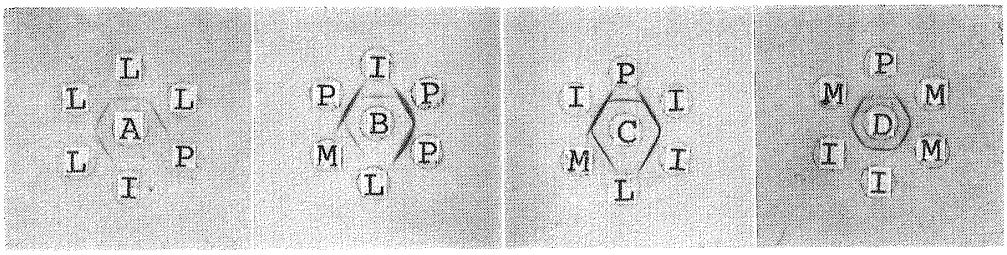

Fig. 3. Immunological Relationship of Human Alkaline Phosphatases from Liver, Placenta, Intestine and Meconium

A, anti-liver alkaline phosphatase antibody; B, anti-placenta alkaline phosphatase antibody; C, anti-intestinal alkaline phosphatase antibody; D, anti-meconial alkaline phosphatase antibody; L, purified liver alkaline phosphatase; P, purified placental alkaline phosphatase; I, purified intestinal alkaline phosphatase; M, purified meconial alkaline phosphatase.

or intestinal alkaline phosphatase. Placental alkaline phosphatase was precipitated by antibodies to placental, intestinal and meconial alkaline phosphatases. Intestinal alkaline phosphatase was precipitated by antibodies to intestinal, placental and meconial alkaline phosphatases. Thus, placental, intestinal and meconial alkaline phosphatases share partially cross-reactive antigenic determinants.

Although a difference of the zinc content of meconial and other enzymes was found, the meconial enzyme was similar to the intestinal enzyme in terms of enzymic and immunological properties.[8,19]

In conclusion, human tissue-specific alkaline phosphatases can be classified into three isoenzymes, liver, intestinal and placental isoenzymes on the basis of the present results. This is in agreement with the conclusion of Lehmann,[20,21] Sussman[17,22] and Stinson[23] on the basis of enzymic and immunological properties. Therefore, these isoenzymes may be synthesized under control of three distinct structural gene codes.

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