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Comparison of Human Alkaline Phosphatase Isoenzymes

STRUCTURAL EVIDENCE FOR THREE PROTEIN CLASSES

By Michael J. McKENNA, Thomas A. HAMILTON and Howard H. SUSSMAN
Laboratory of Experimental Oncology, Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305, *U.S.A.*

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The structural relationships among human alkaline phosphatase isoenzymes from placenta, bone, kidney, liver and intestine were investigated by using three criteria. 1. Immunochemical characterization by using monospecific antisera prepared against either the placental isoenzyme or the liver isoenzyme distinguishes two antigenic groups: bone, kidney and liver isoenzymes cross-react with anti-(liver isoenzyme) serum, and the intestinal and placental isoenzymes cross-react with the anti-(placental isoenzyme) antiserum. 2. High-resolution two-dimensional electrophoresis of the ^{32}P-labelled denatured subunits of each enzyme distinguishes three groups of alkaline phosphatase: (*a*) the liver, bone and kidney isoenzymes, each with a unique isoelectric point in the native form, can be converted into a single form by treatment with neuraminidase; (*b*) the placental isoenzyme, whose position also shifts after removal of sialic acid; and (*c*) the intestinal isoenzyme, which is distinct from all other phosphatases and is unaffected by neuraminidase digestion. 3. Finally, we compare the primary structure of each enzyme by partial proteolytic-peptide 'mapping' in dodecyl sulphate/polyacrylamide gels. These results confirm the primary structural identity of liver and kidney isoenzymes and the non-identity of the placental and intestinal forms. These data provide direct experimental support for the existence of at least three alkaline phosphatase genes.

The human alkaline phosphatases [orthophosphoric monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] comprise a group of membrane-bound glycoproteins that catalyse the hydrolysis of various monophosphate esters *in vitro* (Steck & Wallach, 1970). Multiple molecular forms which are characteristic of selected tissues and cell types have been documented (for reviews see Moss, 1970; Fernley, 1971; Fishman, 1974). The biological role of these isoenzymes has not been defined, but serum concentrations correlate with certain disease entities (Fishman, 1974). Definition of the structural and genetic relationships among such related enzymes is necessary in order to enhance the clinical utility of serum alkaline phosphatase measurements.

In this regard, at least two classes of structurally distinct phosphatases have been defined by peptide 'mapping' and *N*-terminal-sequence analysis (Badger & Sussman, 1976), namely that characteristic of the term placenta and that characteristic of the resting adult liver. However, earlier work suggested that at least three antigenic forms exist (Sussman *et al.*, 1968). Several lines of evidence indicate that the isoenzymes found in liver, bone and kidney are products of the same structural gene: (*a*) activities of liver, bone and kidney phosphatases are all depressed in the autosomal recessive condition hypophosphatasia (Mulivor *et al.*, 1978*a*); (*b*) all three enzymes show similar thermostability profiles (Moss *et al.*, 1972), and response to amino acid inhibition (Mulivor *et al.* 1978*b*); (*c*) all three possess immunochemical cross-reactivity (Boyer, 1963; Sussman *et al.*, 1968; Kamoda & Sakagishi, 1976); and (*d*) all three demonstrate similar electrophoretic behaviour in starch gel after digestion with neuraminidase (Moss *et al.*, 1966; Mulivor *et al.*, 1978*c*). Nevertheless, verification of identical primary structure has not been provided. A similar problem exists with the intestinal isoenzyme. The placental and intestinal isoenzymes have been shown to share some antigenic determinants (Doellgast *et al.*, 1976; Lehmann, 1976) and some enzymic properties including thermostability and inhibitor sensitivity (Mulivor *et al.*, 1978*c*). Despite these similarities, differences in electrophoretic mobility have been demonstrated (Mulivor *et al.*, 1978*c*). These differences could result from variable carbohydrate content or from unique primary structure.

In order to verify the hypothesis that at least three human alkaline phosphatase genes exist, we have partially purified these isoenzymes from human bone, liver, kidney, placenta and duodenum. Comparison of the antigenic, physical and structural properties of these entities supports the three-gene concept: one coding for the placental, one for the intestinal and one for the liver, bone and kidney alkaline phosphatases.

Materials and Methods

Preparation of liver and placental alkaline phosphatase

Alkaline phosphatases from adult liver and full-term placenta were purified as previously described (Badger & Sussman, 1976).

Preparation of kidney, bone and intestinal alkaline phosphatase

Normal kidneys and small intestines were obtained within 24h *post mortem* from cadavers of adult patients who had died of non-malignant non-systemic disease. Bone tissue was collected at autopsy from the vertibular column of a teen-aged male who had died of congestive heart failure, and from vertibular columns, femurs, fibulas, tibias and ribs of 40-week spontaneously aborted foetuses.

Non-parenchymal tissue was removed from the kidneys and excess blood washed away with buffer A (0.01M-Tris/HCl, pH7.4). The surface of the intestinal lumen was washed with water followed by 0.15M-NaCl, and the mucosal cells were scraped into buffer A. Bone was prepared by a modification of the method of Volkin (1955). Bones were minced, homogenized in buffer, and then autolysed at 4°C for 7 days.

Each tissue fraction was extracted in buffer A containing 20% (v/v) butanol, as described by Morton (1954). After 4–6h of gentle agitation at 25°C, the aqueous phase was separated by centrifugation (8000*g*, 30min). The butan-1-ol phase was re-extracted in 25% of the initial volume of buffer, and the two aqueous layers were pooled. Fractional acetone precipitation was conducted as described previously (Sussman *et al.*, 1968). Each enzyme was chromatographed on a column (1.5cm×75cm) of DEAE-cellulose (DE52; Whatman, Maidstone, Kent, U.K.) by using a linear gradient from 0.0 to 0.3M-NaCl in buffer A, pH7.4. The fractions with highest specific activity were pooled. The kidney and intestinal enzymes were further purified on a gel-filtration column (1.5cm×100cm) of Sephacryl S-200 (Pharmacia, Uppsala, Sweden). Final specific activities in units/mg of protein for each enzyme were as follows: placenta, 500–700; intestine, 72; liver, 50–70; kidney, 7; bone, 0.2.

Alkaline phosphatase was assayed by the method of Bessey *et al.* (1946), in which 1 unit of enzyme activity hydrolyses 1 μmol of *p*-nitrophenyl phosphate/min at 37°C. Protein concentration was determined as described by Lowry *et al.* (1951), by using bovine serum albumin as a standard, or estimated by measuring A_{280}.

Immunochemical titration

The antigenicity of each isoenzyme was determined against antibodies raised in sheep against purified placental and liver alkaline phosphatases. By using a double-antibody-precipitation assay (Sussman *et al.*, 1968), approx. 0.005 unit of enzyme was made to react with a range of antisera volumes in buffer B [0.02M-Tris/HCl (pH7.4)/0.002M-$MgCl_2$/0.15M-NaCl] and the activity remaining soluble relative to that after treatment with a control antiserum was used to calculate the percentage that had precipitated.

Labelling with $^{32}P_i$ and two-dimensional polyacrylamide-gel electrophoresis

Approx. 0.01 unit of enzyme activity was immunoprecipitated and radiolabelled as in Hamilton *et al.* (1979*a*). These labelled samples were subjected to sequential isoelectric focusing and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis as described by Wada *et al.* (1977). Some samples were treated with neuraminidase (0.1mg/ml) for 10min at 37°C before immunoprecipitation. Loss of sialic acid does not affect antigenicity.

Peptide 'mapping' of ^{32}P-labelled alkaline phosphatase

Different isoenzymes were subjected to partial proteolysis followed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis by a modification

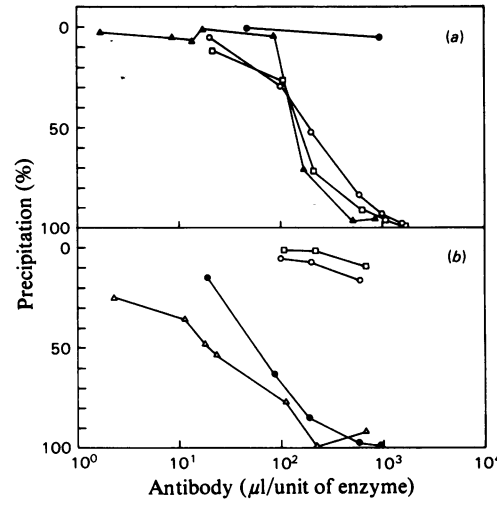

Fig. 1. *Immunochemical characterization of alkaline phosphatase isoenzymes*

Immunoassay was conducted as described in the Materials and Methods section. For each antiserum dilution, a control tube was run containing non-immune serum to provide a 0.0% precipitation value. Between 0.005 and 0.01 unit of enzyme activity was added per assay, and the data have been normalized to antibody/enzyme ratios. The accuracy of the assay is ±5%. (*a*) Anti-(liver enzyme) serum; (*b*) anti-(placental enzyme) serum. Enzymes: △, placental; ●, intestinal; ▲, liver; □, kidney; ○, bone.

of the method of Cleveland *et al.* (1977). Approx. 1–3 units of placental, intestinal, kidney and liver enzymes were pre-digested with neuraminidase as described above. The enzymes were labelled with [^{32}P]P_i as previously described (Wada *et al.*, 1977; Milstein, 1964) and solubilized with 0.125M-Tris/HCl (pH6.8) / 10%(v/v) glycerol / 0.5% sodium dodecyl sulphate/0.005% Bromophenol Blue. The labelled denatured enzymes were treated with various amounts of trypsin or chymotrypsin and incubated at 37°C for 30–60min. The resulting peptide fragments were electrophoresed on 12.5% polyacrylamide gels containing 0.1% sodium dodecyl sulphate. The gels were fixed in 10% (v/v) trichloroacetic acid for 2h, then in 7% (v/v) acetic acid/1% glycerol overnight, dried and then bands located by radioautography at −70°C. Exposure was enhanced by use of Dupont (Wilmington, DE, U.S.A.) 'Lightening-Plus' intensifying screens.

Results and Discussion

We have previously presented evidence describing the non-identical immunochemical behaviour of the isoenzymes from placenta and liver (Sussman *et al.*, 1968). Fig. 1 shows data from an immunochemical titration of each of five different organ isoenzymes by using antisera prepared against either liver (Fig. 1*a*) or placental (Fig. 1*b*) isoenzymes. These results are consistent with results from other laboratories (Kamoda & Sakagishi, 1976; Doellgast *et al.*, 1976). Bone, kidney and liver alkaline phosphatases are all precipitated with anti-(liver isoenzyme) serum and the titration curves for these three enzymes are nearly superimposable. Neither the placental nor the intestinal enzyme cross-reacts with anti-(liver isoenzyme) serum. However, Fig. 1(*b*) shows that anti-(placental isoenzyme) serum effectively precipitates both placental and intestinal enzymes. There is some difference in the behaviour of these two enzymes, since the intestinal enzyme reqires about twice as much antiserum to achieve comparable precipitation. These results suggest that the intestinal and placental isoenzymes do not possess an identical array of antigenic determinants.

Because of this cross-reactivity between specific antisera and isoenzymes, immunological identification alone is insufficient to resolve potential structural differences among these glycoproteins. Figs. 2, 3 and 4 show two-dimensional electrophoretic analysis of

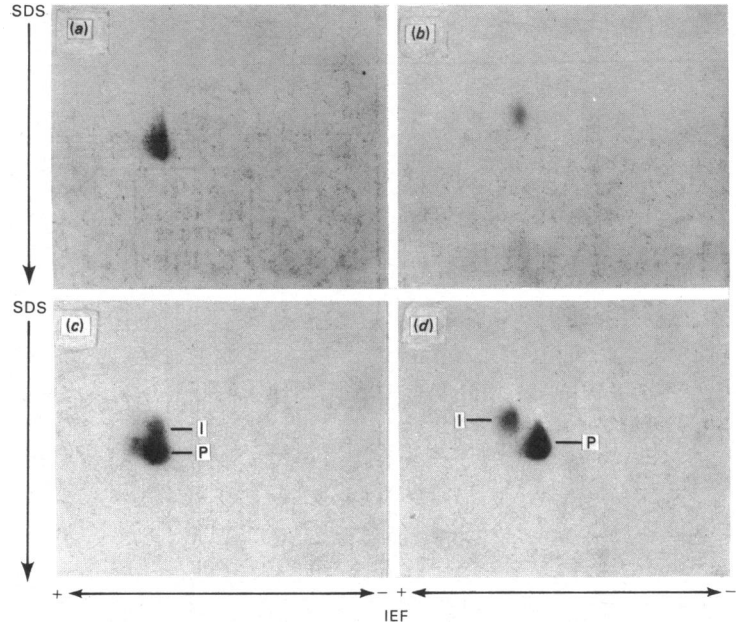

Fig. 2. *Two-L dimensional electrophoretic analysis of placental and intestinal isoenzymes*

For this, 0.01 and 0.05 unit of placental and intestinal alkaline phosphatases, respectively, were immunoprecipitated by using 2μl or 4μl of anti-(placental enzyme) serum, with or without prior digestion with neuraminidase (0.1mg/ml). The isoenzymes were then labelled with [^{32}P]P_i and subjected to two-dimensional polyacrylamide-gel electrophoresis as described in the Materials and Methods section. (*a*) Placental isoenzyme; (*b*) intestinal isoenzyme; (*c*) placental and intestinal isoenzymes; (*d*) placental and intestinal isoenzymes, neuraminidase-digested. Abbreviations: P, placental isoenzyme; I, intestinal isoenzyme. Isoelectric focusing (IEF) is on the horizontal axis and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (SDS) is on the vertical axis.

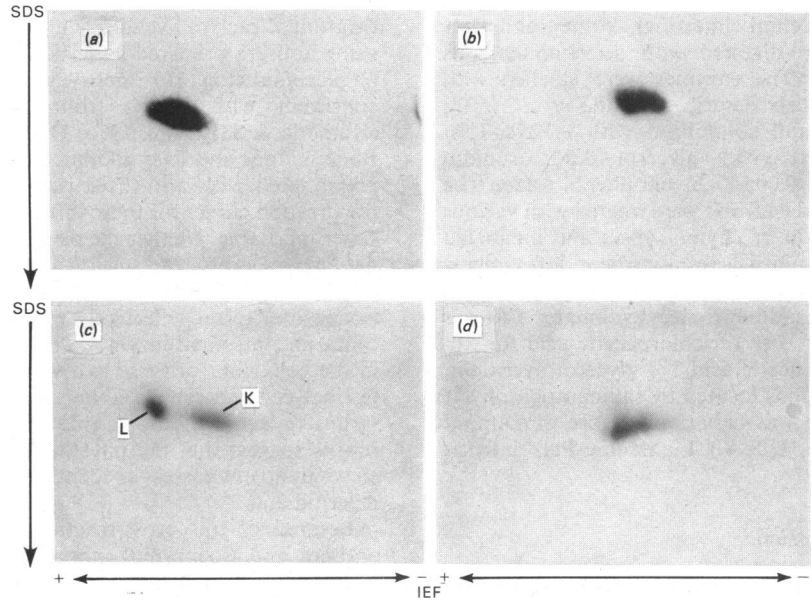

Fig. 3. *Two-dimensional electrophoretic analysis of liver and kidney alkaline phosphatases*
For this, 0.05 unit of either enzyme was treated as described in the legend to Fig. 2, by using 5 μl of anti-(liver enzyme) serum for immunoprecipitation. (*a*) Liver isoenzyme; (*b*) kidney isoenzyme; (*c*) liver and kidney isoenzymes; (*d*) neuraminidase-digested liver and kidney isoenzymes. Abbreviations: L, liver isoenzyme; K, kidney isoenzyme.

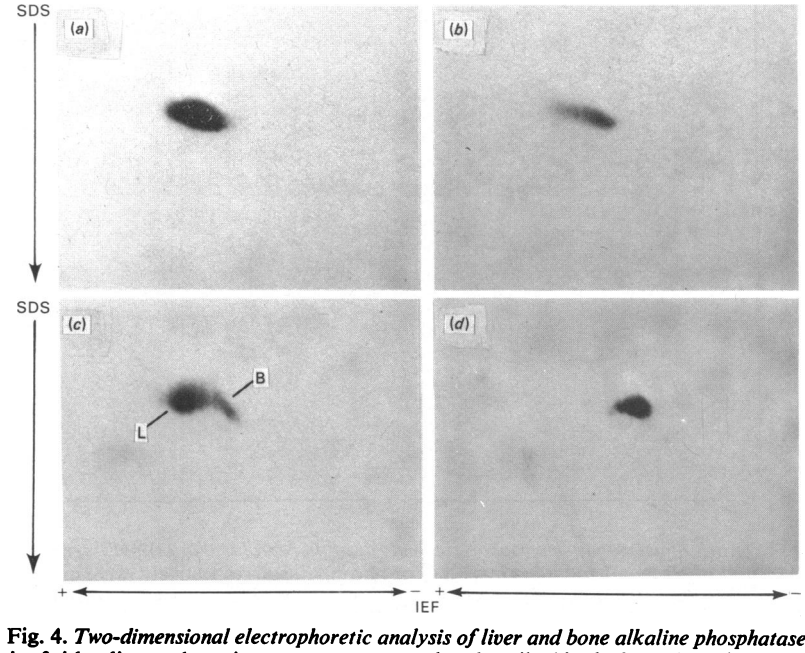

Fig. 4. *Two-dimensional electrophoretic analysis of liver and bone alkaline phosphatases*
For this, 0.05 unit of either liver or bone isoenzyme was treated as described in the legend to Fig. 2, by using 5 μl of anti-(liver enzyme) serum for immunoprecipitation. (*a*) Liver isoenzyme; (*b*) bone isoenzyme; (*c*) liver and bone isoenzymes; (*d*) neuraminidase-digested liver and bone isoenzyme. Abbreviations: L, liver isoenzyme; B, bone isoenzyme.

denatured enzyme subunits that have been covalently labelled with ^{32}P at the active centre. Because glycoproteins exhibit significant microheterogeneity on isoelectric focusing, the individual enzyme spots have characteristic patterns which make the identification of each more readily achieved. In addition, some alkaline phosphatases are known to be sialoglycoproteins (Kamoda & Sakagishi, 1978); hence we have used neuraminidase digestion before electrophoretic analysis in order to evaluate this specific aspect of carbohydrate structure. Finally, each enzyme was immunoprecipitated before ^{32}P labelling. This defines the relationship between the enzyme activity measured in the immunochemical assay and the subunit spots detected in the two-dimensional gel.

Fig. 2 explores the physical relationship between the cross-reacting placental and intestinal enzymes. Figs. 2(*a*) and 2(*b*) are gels of the placental and intestinal phosphatases respectively. The former had an isoelectric point (pI) of 5.9–6.0, and an apparent subunit molecular weight (M_r) of 64000, and the latter has an identical pI but a slightly higher M_r of 70000. The relationship is most easily seen in Fig. 2(*c*), where both subunits are run in the same gel. Fig. 2(*d*) demonstrates the effect of neuraminidase digestion on the two isoenzymes. In this gel the placental isoenzyme is shifted to a more basic pI and a lower M_r, whereas the intestinal isoenzyme is not measurably affected by this treatment. Others have measured the sialic acid content of both placental and intestinal enzymes, and demonstrated that the intestinal enzyme lacks sialic acid (Kamoda & Sakagishi, 1978). However, the results described here also suggest that the absence of sialic acid is not the only structural difference between these two isoenzymes.

The relationships between bone and liver and kidney and liver enzymes are similarly demonstrated in Figs. 3 and 4. Again all three enzymes show characteristic spot patterns. However, this set of enzymes is more readily distinguished by differences in pI than in M_r. Liver enzyme is the most acidic of the three, bone enzyme is only slightly more basic, and kidney enzyme is the most basic of all. Comparing Figs. 3(*c*) and 4(*c*) shows that liver and bone enzymes overlap significantly, whereas liver and kidney enzymes are distinct entities which are totally resolved in this gel. Figs. 3(*d*) and 4(*d*) clearly demonstrate that all three enzymes represent a range of sialylation and all three distinct forms can be reduced to a single coincident spot. These data are consistent with previous work using electrophoretic mobility on native starch gel of crude enzyme preparations with or without neuraminidase digestion (Mulivor *et al.*, 1978*c*). A similar relationship between human liver and milk alkaline phosphatases has been previously demonstrated (Hamilton *et al.*, 1979*b*). Indeed, isoelectric microheterogeneity of alkaline phosphatases from a variety of mammalian sources has been shown to be principally due to differences in sialic acid content. It seems likely that other oligosaccharide differences may exist between the enzymes considered here. However, because these residues carry no net charge, they are not easily resolved on these gels.

As a third approach to the structural relationships among these enzymes, we used partial proteolysis of ^{32}P-labelled subunits and analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and radioautography. Although only active-centre-containing peptides are detected, this technical approach does provide direct information about primary structure. Fig. 5 shows gels of the placental, liver, intestinal and kidney isoenzymes which have been partially digested with trypsin (Fig. 5*a*) and chymotrypsin (Fig. 5*b*). Bone alkaline phosphatase was not subjected to this form of analysis owing to insufficient material and enzyme purity. These gels verify previous peptide-'mapping' studies which demonstrated unique primary structures for placental and liver isoenzymes (Badger & Sussman, 1976). Placental and intestinal phosphatases behaved distinctly differently, despite their antigenic identity, in response to both trypsin and chymotrypsin. The intestinal enzyme is quite resistant to proteolysis, though trypsin digestion does produce two peptides (Fig. 5*a*, lane 2) which are distinctly different from any peptides seen with the placental form (lane 1). With both trypsin and chymotrypsin, the kidney (lane 3) and liver (lane 4) enzymes give identical patterns. The different intensity of labelling makes some bands more difficult to detect in photographic reproduction of the original radioautograph. However, they can be seen in more intense exposures, where resolution of the majority of the bands is lost (results not shown).

Most investigations of the structural and functional relatedness of human alkaline phosphatase isoenzymes have generally used immunological and enzymic data as well as native electrophoretic mobility for purposes of isoenzyme identification (Fishman, 1974; Mulivor *et al.*, 1978*c*; Sussman, 1978). Each of these criteria is dependent on multiple experimental variables whose contribution to the total enzyme behaviour cannot be easily resolved to provide accurate characterization. However, the criteria applied in the present study measure physical constants of subunit molecular weight and pI and present the only direct evidence available comparing primary structure of alkaline phosphatases from human kidney and intestine. We consider that these data provide strong support for the concept that at least three different structural genes code for the different isoenzymes evaluated here.

If the kidney, liver and bone alkaline phosphatases are coded by the same structural gene, their non-identical carbohydrate moieties (as indicated by variable sialic acid content) suggest that each differentiated tissue from which these enzymes were prepared

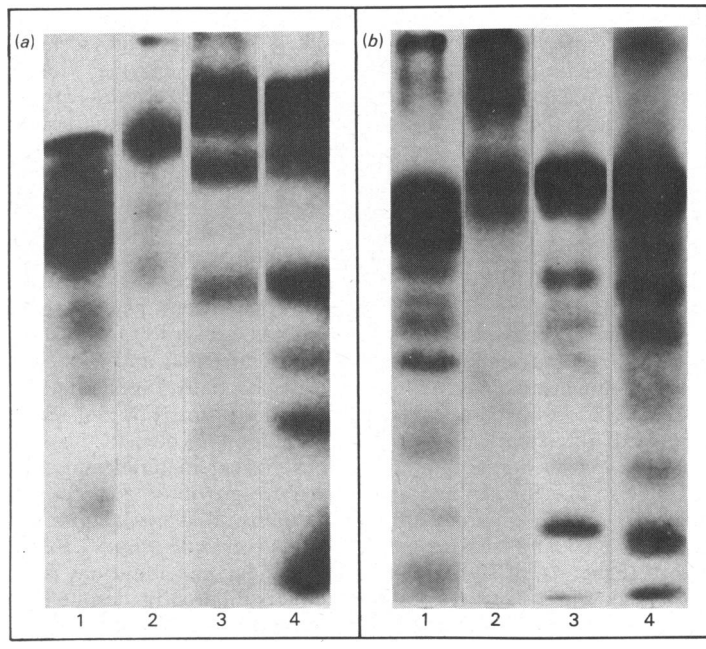

Fig. 5. *Active-centre-peptide 'mapping' by partial proteolysis*

Isoenzymes from placenta, intestine liver and kidney were labelled for peptide 'mapping' as described in the Materials and Methods section. The samples were then digested at 37°C with the indicated concentrations of either trypsin or chymotrypsin for 30–60min. The digestion was stopped by addition of 25% sodium dodecyl sulphate and 5% mercaptoethanol and boiled for 2min. The samples were then subjected to electrophoresis in 12.5% polyacrylamide gels. (*a*) Trypsin digestion: lane 1, placental isoenzyme, 0.2mg/ml, 60min; lane 2, intestinal isoenzyme, 0.2mg/ml, 60min; lane 3, liver isoenzyme, 0.2mg/ml, 30min; lane 4, kidney isoenzyme, 0.2mg/ml, 30min. (*b*) Chymotrypsin digestion: lane 1, placental isoenzyme, 0.01 mg/ml, 60min; lane 2, intestinal isoenzyme, 1.0mg/ml, 60min; lane 3, liver isoenzyme, 0.01 mg/ml, 60min; kidney isoenzyme, 0.01 mg/ml, 60min. Electrophoresis is from top to bottom.

must express a unique complement of enzymic machinery for post-translational glycosylation of membrane-bound and secreted proteins. Similar observations with other glycoproteins have been reported (Hercz *et al.*, 1978; Bramwell & Harris, 1978).

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