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PURIFICATION OF PHENYLALANINE HYDROXYLASE FROM HUMAN ADULT AND FOETAL LIVERS WITH A MONOCLONAL ANTIBODY

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Phenylalanine hydroxylase from adult and foetal livers was purified by single step monoclonal antibody affinity chromatography. From adult and foetal livers, about 1280- and 1450-fold purified enzymes were obtained with 37% and 23% yield, respectively. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the resultant adult enzyme showed an essentially single band with an apparent molecular weight of 49K. On the other hand, two subunits (molecular weights 52K and 49K) were observed from the foetal enzyme. Molecular weights of the native adult and foetal enzymes as determined on Sepharose CL-6B column chromatogram were 150K and 160K, respectively. It was clear that adult and foetal liver phenylalanine hydroxylases were different proteins having different subunit molecular weights.

Phenylketonuria is a human disease characterized by a lack of phenylalanine hydroxylase (EC.1.14.16.1) in the liver. Most of current knowledge on this enzyme has come from studies on the enzyme from rat liver. Because of its instability and rapid disappearance of the activity after death (1), little knowledge on the human enzyme has been obtained and there are some points of disagreement in its characterization. For example, it was reported that phenylalanine hydroxylase had a M.W. of 108K by Woo et al. (2), 275K by Choo et al. (3) and 165K by Abita et al. (4). And it was reported by Woo et al. (2) that human adult liver contained a single phenylalanine hydroxylase with subunit M.W. of 54K the same as that of foetal enzyme. Recently, human

Abbreviations: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ELISA, enzyme linked immunosorbent assay; M.W., molecular weight

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phenylalanine hydroxylase cDNA clones have been isolated and the predicted amino acid sequence resulted in a protein with a subunit M.W. of 51.9K (5).

In the present work, we have isolated a monoclonal antibody to phenylalanine hydroxylase by using a partially purified enzyme as an antigen and succeeded in rapid purification of the enzyme by a single step monoclonal antibody affinity chromatography. Moreover, it was proved that adult and foetal enzymes had different subunit M.Ws..

Materials and Methods

Monoclonal antibody to human phenylalanine hydroxylase: Human phenylalanine hydroxylase, partially purified on Blue Sepharose (Bio Rad) and HPLC (IEX-545, Toyo Soda MFG. Co. Ltd.) was used as an antigen, whose purity was 10-20% as determined densitometrically by PAGE. The hybridoma secreting monoclonal antibody to human phenylalanine hydroxylase was prepared according to the procedure of Köhler and Milstein (6). Monoclonal antibody to the enzyme was selected by a combination of ELISA and phenylalanine hydroxylase inhibition assay. Briefly, the inhibition assay was performed as follows: the culture sup. of ELISA-positive hybridoma was added to 10^5g sup. of human liver homogenates and the antigen-antibody complex was then removed with IgG sorb (THE ENZYME CENTER) by centrifugation and the enzyme activity in the resultant sup. was assayed. A large amount of the monoclonal antibody to phenylalanine hydroxylase was purified from mouse ascitic fluids on DEAE Affi Gel Blue (Bio Rad) chromatogrophy as described by Bruck et al. (7).

Purification of phenylalanine hydroxylase on immunoadsorbent column: Human liver was homogenized in 4 vol. buffer containing 50mM sodium phosphate (pH 7.2), 10mM phenylalanine, 10% glycerol and 1mM dithiothreitol and centrifuged at 10^5g for 30min. at 4°C. The supernatant was applied to the column in which Affi Gel 10 (Bio Rad) fixed with phenylalanine hydroxylase-directed monoclonal antibody was packed. The column was washed thoroughly with 50mM Tris-HCl (pH 7.2) containing 1M NaCl, 10mM phenylalanine, 10% glycerol and 0.1% Tween 20, then phenylalanine hydroxylase was eluted with 50mM acetic acid containing 10mM phenylalanine, 10% glycerol and 0.1% Tween 20 and the eluates were then immediately neutralized with 1M NaOH and 1M Tris-HCl (pH 7.2).

Others: Phenylalanine hydroxylase activity was measured as described by Woo et al. (2) or Choo et al. (8). Proteins were determined by the dye binding method (Bio Rad Protein Assay). SDS-PAGE was done according to Laemmli (9). Gel filtration on Sepharose CL-6B (Pharmacia) was done as described in the legend of Fig. 4.

Results

Characterization of monoclonal antibody 21-5-12: One hybridoma secreting monoclonal antibody to human liver phenylalanine hydroxylase was established from a cell of the mouse immunized with 250μg of partially purified antigen. The monoclonal antibody 21-5-12 was IgG_1 (light chain was κ) and it directly inhibited the phenylalanine hydroxylase by 80%. But it did not cross-react with the phenylalanine hydroxylase from the liver of mouse, rat or monkey. Therefore, it is likely that 21-5-12 antibody recognizes the specific site for

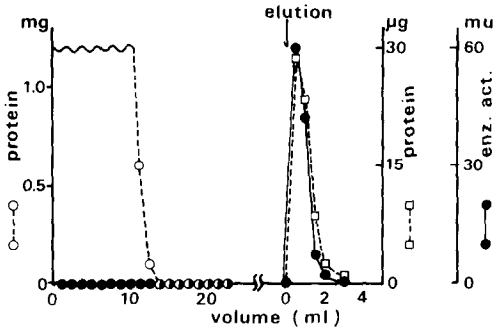


Fig. 1. Elution profile of human adult phenylalanine hydroxylase from monoclonal antibody affinity column. The 10^5g sup. of the homogenate of 2.5g adult liver was applied on an immunoadosorbent column (0.7 x 0.4 cm) and washing and elution were done as described in Materials and Methods. ●, phenylalanine hydroxylase activity. ○, □, amounts of protein. One unit of activity was defined as 1μmol of tyrosine formed/min. at 25°C.

human enzyme which affects the enzyme activity by binding to the 21-5-12 antibody.

Purification of human phenylalanine hydroxylase: 10^5g sup. of the homogenate of 2.5g human adult liver was applied on a monoclonal antibody affinity column. The column was extensively washed and then phenylalanine hydroxylase was eluted with the solvent system described in Materials and Methods. The eluate was neutralized immediately. As shown in Fig. 1, purification on this immunoadsorbent column was very effective. None of the phenylalanine hydroxylase was detected in the flow-through fractions and it was sharply eluted with the acid solution. The resultant enzyme was essentially pure, as described below. When human foetal liver was used as a starting material, the same results were obtained.

The results of purification of phenylalanine hydroxylases from human adult and foetal livers are summarized in Tab. 1. By this single step procedure, the enzymes from adult and foetal livers were purified by 1280-fold with 37% yield and 1450-fold with 23% yield, respectively.

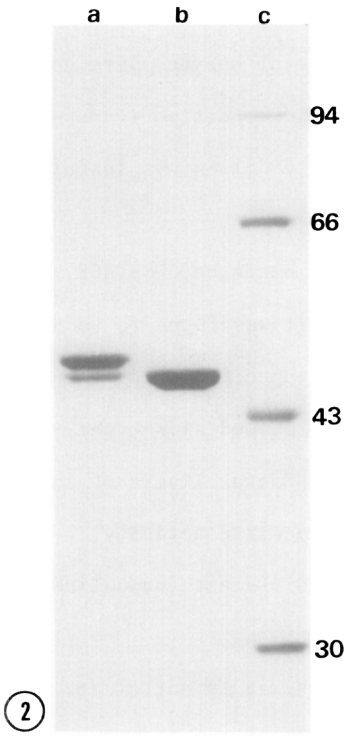
Physical properties: SDS-PAGE of adult phenylalanine hydroxylase showed an essentially single band, which had an apparent subunit M.W. of 49K. On the other hand, foetal phenylalanine hydroxylase had two subunits; one having a M.W. of 52K (major) and the other 49K (minor) (Fig. 2 lane a and b).

Table 1
Purification of human adult and foetal liver phenylalanine hydroxylase

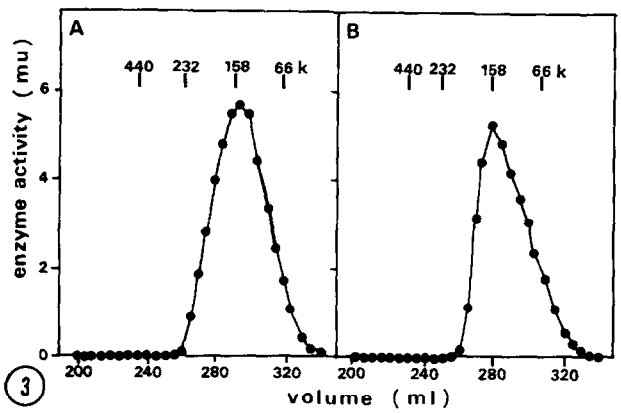
		total activity (mu)	total protein (mg)	specific activity (mu/mg)	purification (-fold)	yield (%)
*adult	10^5g sup.	292	213	1.37	1	100
	elution	108	0.0614	1760	1280	37
*foetal	10^5g sup.	223	202	1.01	1	100
	elution	50.2	0.0314	1600	1450	23

*Adult and foetal livers (2.5g each) were used as the starting materials.

The native M.W. of adult and foetal phenylalanine hydroxylases was determined by gel filtration on a Sepharose CL-6B column (Fig. 3). Consequently,



(2)



(3)

Fig. 2. Phenylalanine hydroxylase purified on an immunoadsorbent column was analyzed by SDS-PAGE. Lane a, foetal enzyme; lane b, adult enzyme; lane c, M.W. standards (94K, phosphorylase b; 66K, bovine serum albumin; 43K, ovalbumin; 30K, carbonic anhydrase).

Fig. 3. Sepharose CL-6B chromatography of purified adult and foetal liver phenylalanine hydroxylase. Purified enzymes with M.W. standards on an immunoadsorbent column were loaded on a Sepharose CL-6B column (2.6 x 88 cm) equilibrated with Tris-HCl (pH 7.2) containing 1M NaCl and the column was developed with the same buffer at 4°C at a speed of 6ml/h. ● , phenylalanine hydroxylase activity (A; adult enzyme, B; foetal enzyme). Arrows in A and B represent elution patterns of ferritin (440K), catalase (232K), aldolase (158K) and bovine serum albumin (66K), respectively.

only one peak was observed under the conditions described in the Fig. 3 legend and the native M.Ws. of adult and foetal enzymes were estimated to be about 150K and 160K, respectively.

Discussion

Human liver phenylalanine hydroxylase was very labile as compared with that of rat. In this aspect purification of human enzyme on an antibody affinity column was extremely effective, because one step rapid purification procedure is expected. Human enzyme was so labile that only partially purified one was available as an antigen, but we could successfully obtained the monoclonal antibody to this antigen.

As shown in Tab. 1, the yields of both adult and foetal enzymes were not satisfactory because of their instability under the acidic condition (unpublished data, 2). The enzyme bound to an antibody affinity column was inactivated during its elution with the acidic solution.

The enzyme purified from foetal liver indicated two bands on SDS-PAGE at M.Ws. of 52K and 49K, as shown in Fig. 2. The 52K subunit was found to be not ascribed to the contaminant protein, because both of the specific activities of phenylalanine hydroxylases of adult and foetal livers showed almost the same value (Tab. 1) and also all of the enzymes from the foetal livers of four individuals possessed two subunits and almost the same specific activity, although the ratio between the 52K and 49K subunits was different (unpublished data).

The reason that the enzymes have different subunit M.Ws. is not clear. Recently, it was reported that the gene of human liver phenylalanine hydroxylase was cloned and the gene was present in only one copy per haploid genome (5,10). We also observed that the amino acid sequences of both enzymes resembled each other (in preparation). It is likely, therefore, that the different subunit M.Ws. of foetal enzyme is due either to different processing of the protein or different splicing of precursor mRNA. But some enzymological parameters of both enzymes were almost identical (in preparation).

The different values for the native M.W. of phenylalanine hydroxylase have been reported by different investigators (2,3,4). As shown in Fig. 3, we observed the native M.Ws. of adult and foetal liver enzymes to be about 150K and 160K, respectively, which suggested that both enzymes are trimers.

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