"protein sparing" effects of human growth hormone in man are demonstrated.

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Studies on Mammalian Tyrosinase. II. Chemical and Physical Properties Of Fractions Purified by Chromatography.* (24749)

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Our recent reports have dealt with isolation of tyrosinase from Harding-Passey mouse melanoma(1) and with its purification on diethylaminoethyl (DEAE) cellulose ion exchange columns(2). At least 3 chromatographically distinct components with tyrosinase activity were detected and designated I, II and III in the order of movement on and emergence from the column(2). It is the purpose of this paper further to describe highly purified mammalian tyrosinase in respect to some of its chemical and physical properties, and to report a rapid purification procedure using hydroxyapatite ion-exchange columns.

Methods. The highly purified tyrosinase samples used in this study were prepared by column chromatography using DEAE cellulose(2) or hydroxyapatite as the ion-exchange agent. Hydroxyapatite, prepared according to Tiselius *et al.*,(3), has been found to remove most of the impurities present in crude fractions of soluble mammalian tyrosinase(1) without adsorption of the enzyme. For these studies 1 x 11 cm, or, for preparative scale, 2.2 x 13 cm columns have been employed. Initially, development was started with pH 6.8 phosphate buffer, 0.001 M, followed by gradient elution to concentrations of 0.5 M phosphate buffer, pH 6.8. After it became clear that most of the tyrosinase activity passed through the column without absorption, simple elution with starting buffer, pH 6.8, 0.001 M, was utilized. Protein in effluent fractions was analyzed by the Folin-Lowry reaction (4). Tyrosinase activity was detected as previously described(2). After location of tyrosinase activity, fractions were combined, dialyzed from 4 to 6 hours in the cold, and lyophilized. Specific activity was determined on dried material as previously reported(2). Nitrogen for this calculation was estimated by the method of Schaffer and Sprecher(5). Ultracentrifuge studies were carried out on solutions of tyrosinase in pH 6.5, 0.05M cacodylate buffer. Aliquots containing 1.18 and 2.25 mg of protein nitrogen/ml were placed in the Spinco model E ultracentrifuge and subjected to centrifugal forces of 260,000 x g.

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Photographs were made at 16 and 32 minute intervals over periods up to 5 hours. Copper was determined on wet-ashed samples of highly purified tyrosinase colorimetrically, using diethyldithiocarbamate as prescribed by Gubler *et al.*(6). Ashing was accomplished by digesting aliquots of tyrosinase with sulfuric acid at 450° in a sealed tube(5). Recovery of copper which had been added to protein solutions was 89-94% effective by this procedure. The per cent copper contained in tyrosinase was estimated on the basis of dry weight determined on aliquots placed in weighing vessels and dried to constant weight in a 110° oven. Hydrolysis of material (purified by either hydroxyapatite or DEAE-chromatography, then dialyzed 6 hours in the cold) was carried out with 6N-HCl in sealed tubes at 110°C for 16 hours. Aliquots equivalent to 0.4 to 0.6 mg protein were chromatographed on Whatman #1 paper with butanol: formic acid: H_2O (75:15:10) then lutidine: H_2O (60:40). Amino acid spots were detected by spraying with ninhydrin. Identification of individual amino acids was by comparison with chromatograms of known amino acid mixtures.

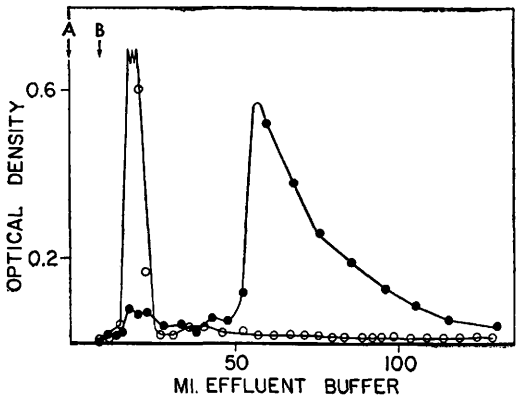

FIG. 1. Chromatography of 24 mg of tyrosinase "R_3"(2) on hydroxyapatite ion exchange agent in 1 × 11 cm column. Collection after one hold-up volume (9 ml), 2 ml fractions. ○—○ Tyrosinase assay on 0.1 ml aliquots in terms of melanin produced after incubation with 1 mg of 3,4-dihydroxyphenylalanine (dopa) in 3 ml pH 6.8 buffer for 45 min. O.D. measured at 400 mμ, Coleman spectrophotometer. In area under jagged line, melanin precipitated from assay reaction mixtures. ●—● Folin-Lowry reaction(4) on 0.2 ml aliquots measured at 700 mμ. A. 0.001 M phosphate buffer, pH 6.8. B. Gradient to 0.1 M phosphate buffer, pH 6.8 through a 50 ml mixing chamber.

Results. Fig. 1 shows the results obtained when 24 mg of crude tyrosinase preparation "R_3"(1) was placed on a hydroxyapatite column and subjected to gradient elution. A highly active tyrosinase peak came through the column after one hold-up volume of approximately 9 ml, whereas most of the protein impurities were adsorbed to the ion-exchange agent. Specific activity of the material in the active peak was 100 units/mg nitrogen which represents a 40 to 50-fold purification of tyrosinase from tumor homogenates(1). In repeated experiments, 70% of the activity placed on the column was recovered.

Using a larger column (2.2 x 13.5 cm) and a larger quantity of starting material (950 mg), a chromatogram similar to Fig. 1 was obtained. Considerable protein and a brown-colored material which had slight tyrosinase activity could also be eluted with 0.5 M phosphate buffer at pH 6.8.

An attempt was made to characterize material analogous to the tyrosinase peak shown in Fig. 1 with respect to its behavior on DEAE cellulose, using a column (1 x 67 cm) containing 6 g of DEAE with a hold-up volume of 30 ml. The material (103 mg), after dialysis and lyophilization, was put on the column in 0.005 M, pH 6.0 phosphate buffer. Gradient elution was performed through a 50 ml mixing chamber at room temperature. Eluting phosphate buffers ranged from 0.005 M to 0.12 M and from pH 6.0 to 8.0. Fig. 2 shows that active material which was not adsorbed to hydroxyapatite (Fig. 1) was resolved into component II and III by chromatography on DEAE(2). Component I was apparently retained by the acidic hydroxyapatite. As previously mentioned, a crude active fraction, probably component I, was eluted from the hydroxyapatite with 0.5 M phosphate buffer at pH 6.8. This fraction was not studied on DEAE, since the activity was too small.

Solutions of tyrosinase containing 1.18 to 2.25 mg of protein nitrogen per ml of cacodylate buffer were subjected to centrifugation at 260,000 x g. Previous preparation of sample had involved dialysis for a period of 6 hours on a rocking dialysis apparatus. A single small peak was observed, but some ma-

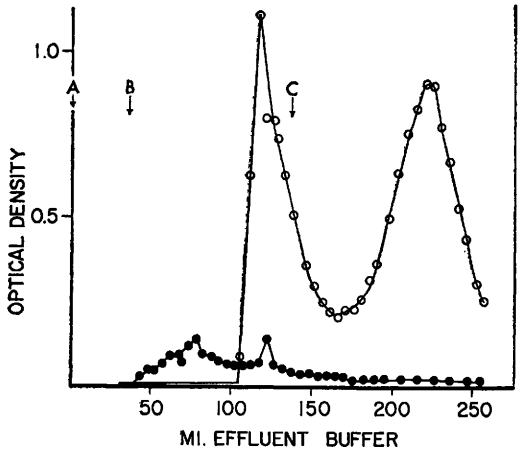

FIG. 2. Chromatography of partially purified tyrosinase on DEAE cellulose ion exchanger (6 g in a column 1 × 67 cm). Material applied to column: 105 mg of active fraction obtained after chromatography on a hydroxyapatite column as in Fig. 1. Collections after one hold-up volume (30 ml), 2.5 ml fractions. ○—○ Tyrosinase assay on 0.2 ml aliquots in terms of melanin produced after incubating with 1 mg dopa in 3 ml, pH 6.8 buffer for 30 min. O.D. measured at 400 mμ, Coleman spectrophotometer. ●—● Folin-Lowry(4) reaction on 0.1 ml aliquots measured at 700 mμ. A. 0.005 M phosphate buffer, pH 6.0, plus 0.05 M NaCl. B. 0.05 M phosphate buffer, pH 8.0, with gradient to 0.08 M phosphate, pH 8.0, through a 50 ml mixing chamber. C. Gradient change to 0.12 M phosphate, pH 8.0. Tyrosinase emerging at 105 to 140 ml effluent is equivalent to Component II; that emerging at 180 to 250 ml effluent equivalent to Component III of earlier studies(2).

terial did not sediment. After 5 hours of centrifugation, the upper two-thirds of the cell contents were carefully withdrawn with a needle, and upper and lower fractions were assayed for tyrosinase activity and nitrogen content. All activity and 86% of protein nitrogen had been sedimented to the bottom of the cell. Thus the purity was probably no better than 86%.

Copper determinations were carried out on various fractions obtained from tumor homogenates of mouse melanoma, and on highly purified samples. Previous to copper analyses, preparations were dialyzed for 96 hours against 250 ml of glass-distilled H_2O in seamless cellulose tubing which had been washed with several changes of glass-distilled water previous to use. At the end of this time, copper and nitrogen content of material inside the bag was analyzed and the tyrosinase activity measured. Results are shown in Table I. The value of 0.25% for purified fractions is comparable with values obtained for plant tyrosinase(7). However, 95% of the activity and 60% of the nitrogen which had been placed in the dialysis sack was lost in the case of the highly purified tyrosinase fractions. Nitrogen determinations on lyophilized dialyzate indicated that nitrogenous material had passed through the sack. The dialyzate also gave a strongly positive response to Folin-Lowry reagent. Neither the material inside the dialysis bag, the concentrated dialyzate, nor mixtures of the 2 were active. Addition of copper to the assay vessels had no reactivating effect. Lack of material has prevented further experiments to determine the reason for loss of activity under these conditions. With such an inactivation one cannot decide whether the measured copper content of the preparation is an integral part of the enzyme.

Qualitative amino acid analyses on highly purified tyrosinase samples showed presence of the following amino acids: leucine and/or isoleucine, valine, alanine, proline, glutamic and aspartic acid, lysine, glycine, threonine, serine, and cysteic acid. The latter was probably due to oxidation of cystine during hydrolysis. Very slight traces of phenylalanine, tyrosine, methionine, arginine and histidine were present. The presence or absence of tryptophan could not be evaluated. The low content of aromatic amino acids is consistent with observations that tyrosinase has a low specific absorption at 280 mμ(2). No qualitative difference in amino acid content of different tyrosinase active components obtained from DEAE(2) was detected. Since the ultracentrifuge study indicates our best prepa-

TABLE I. Copper and Nitrogen Content of Mammalian Tyrosinase Preparations after Prolonged Dialysis.

Sample	Dry wt, mg	Copper, μg	Copper, %	Nitrogen, %
Tumor homogenate*	4.08	.8	.02	11.5
R_3*	3.61	3.25	.09	18.3
Component II†	.45	1.75	.22	
" III†	.40	1.55	.25	12

* For preparation of R_3 from crude homogenates of Harding-Passey Mouse Melanoma see reference (1).

† Component II and III, Fig. 2.

rations are still impure, the important observation is the virtual absence of certain amino acids since some of the amino acids detected must have been derived from impurities. The very low content of tyrosine probably accounts for the low Folin-Lowry color obtained in the area of tyrosinase activity in Fig. 2.

Summary. A procedure for obtaining a 50-fold purification of mammalian tyrosinase on hydroxyapatite has been described. Various highly purified samples of tyrosinase have been studied in the ultracentrifuge. The preparations were not homogeneous. The per cent of copper present in highly purified fractions was shown to be 0.22 to 0.25%, although it cannot be concluded this is an integral part of the enzyme, since prolonged dialysis inactivates the enzyme and the addition of copper did not restore the activity. Qualitative analysis for amino acids by paper chromatography indicated leucine and/or isoleucine, valine, alanine, proline, glutamic and aspartic acid, lysine, glycine, threonine, serine, and cysteic acid (probably from cystine) were present in significant amounts. Traces of phenylalanine, tyrosine, methionine, arginine, and histidine were also present.

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Properties and Structure of Lipoproteins.* (24750)

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A knowledge of properties and structure of serum lipoproteins is important for understanding of their physiological functions. During recent years there have been efforts to relate certain lipoprotein classes to the pathogenesis or prediction of atherosclerosis. So much controversy has arisen that it is imperative that extensive studies of lipid-protein complex be carried out to clarify their structural relationship to their function. Lipid composition and distribution of serum lipoproteins have been studied extensively(1). However, relatively little work has been done on protein moiety of these lipoproteins. The protein portion of lipoproteins may be studied while combined with lipid(2) or following removal of the lipid fraction(3). Extraction of serum lipoproteins with ether removes a high percentage of triglycerides and cholesterol, but considerable phospholipid remains unextracted. Almost complete removal of all lipid materials may be achieved with extraction of lipoproteins with ether-alcohol mixtures(2). In the present work lipoprotein fractions from various sources were extracted with ether, and properties of nonextractable material studied.

Methods and materials. 1. Extraction of low density lipoproteins with ether. Lipoproteins from various sources were prepared by the method of Gofman, Lindgren, and associates(1,4) as follows: Lipoproteins of density less than 1.063 g/ml were obtained by preparative ultracentrifugation in Spinco Model L Ultracentrifuge at 30,000 rpm for 24 hrs. Three volumes of serum were mixed with 6 volumes of sodium chloride solution of density 1.0918 g/ml to final density of 1.063 g/ml. After preparative ultracentrifugation, the top ml of solution was removed as lipoprotein

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