Preparation of ^{14}C-3-Hydroxyproline from ^{14}C-Proline by Peroxidation.*

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SUMMARY

Conditions are described for the treatment of proline with hydrogen peroxide, ascorbic acid, and ferrous ions to produce 3-hydroxyproline in 5-6% yield. Several reaction products are formed, but they can be separated chromatographically to yield pure trans-3-hydroxyproline. Synthesis of the radioactive amino acid from ^{14}C-L-proline yields the pure isomer with specific activity identical to that of the starting proline. Identity of the desired product was established by chromatography, electrophoresis, and mass spectrum.

key words: 3-hydroxyproline, preparation, purification

INTRODUCTION

3-Hydroxyproline (3-Hyp) is a relatively rare positional isomer of 4-hyp. It was first recognized (1,2) in bovine tendon collagen, where its residue frequency is approximately 1-2 per thousand amino acids (3). In other species of collagen, e.g., renal basement membrane collagen, 3-hyp residues occur at much higher frequency -- 20 per thousand (4); but it is always a minor residue compared to 4-hyp. During the biosynthesis of collagen, 3-hyp --like 4 hyp-- is generated from proline residues as a posttranslational event (5,6).

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*Dedicated to the memory of Elijah Adams, who has made a lasting impression on the author. This work was started under his supervision shortly before his death.

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Limited availability of free 3-hyp has been a problem for studies of the amino acid itself. Perhaps reflecting its scarcity in the collagens and absence from proteins in general, the compound does not appear to be present at greater than trace levels in most free amino acid pools. It has been isolated on a small preparative scale from seeds of a tropical plant, Delonix regia, where it occurs in the amino acid pool (7). The same source has served (8) for the isolation of isotopically labeled material following endogenous biosynthesis from added, labeled proline. However, isolation of 3-hyp from these seeds is tedious and attended by poor and variable yields. Furthermore, the ability to prepare high specific activity material biosynthetically is compromised by large dilution of the added, labeled proline by proline in the seeds. A number of chemical synthetic methods for 3-hyp have been described (2, 9-14) but these, too, are tedious and unsatisfactory from the points of view of yield, purity, and isomer selectivity; they produce mixtures of all four geometrical isomers.

Our interest in the metabolism of 3-hyp in the rat led us to explore means of preparing labeled 3-hyp. We report here a simple and reproducible method for accomplishing this. It utilizes a chemical hydroxylation of proline with a reagent containing hydrogen peroxide, ferrous ion, and ascorbic acid.

MATERIALS AND METHODS

U-^{14}C-Proline (Sp. act. 250 mCi/mmole) was purchased from ICN and purified by ion-exchange chromatography on Dowex 50X2 (H+) with 0.5N HCl elution. Unlabeled trans-3-Hydroxyproline and trans-(3-hydroxy-G-^{3}H)-L-Proline were obtained as previously noted (15); the ^{3}H-tracer had a sp. act. of 5.32 mCi/mmole. Unlabeled L-proline was from Sigma Chemical Co., St. Louis. Ascorbic acid, hydrogen peroxide (30%), and Norit A were from Fisher Scientific Co. AG 50WX2 (200-400 mesh) resin and AG 11A8(50-100 mesh) ion-retardation resin were from BioRad, Richmond, California. All other chemicals were of the best commercially available grades.

Paper electrophoresis was carried out at pH 1.85 with 200 volts/cm (16). Amino acid analysis was performed as described earlier (4); with this method, trans - and cis-3-hyp are eluted at 60-65 minutes and 105-110 minutes, respectively; trans-4-hyp is eluted at 70-75 minutes. Optical rotations were determined in a Bendix NPL Polarimeter. Electron impact mass spectrum of 3-hyp, after derivatization to its trifluoroacetylated butyl ester (17), was determined with a Du Pont 21-490 mass spectrometer operating at 70 ev in conjunction with a Du Pont 094 data system for data acquisition and processing.

Reaction Conditions

Preliminary experiments indicated that the concentration of ascorbic acid was an important determinant of yield. This variable was investigated in reaction mixture of 4 milliliters containing 10 mM HCl, 20 mM proline, 20 mM ferrous ammonium sulfate, varying levels of ascorbic acid, and 50 mM hydrogen peroxide. The peroxide was added last. After ten minutes at 37° C, 2 ml of a suspension of AG 50 resin in 0.1N HCl was added to bind the 3-hyp and other amino acids in the reaction mixture. A small quantity of ^{3}H-3-hyp was also added as tracer.

The resin containing bound reaction products was transferred to a small column of the same resin to form a column of total dimensions, 8 x 0.9 cm. Following a column wash with 25 milliliters of water, amino acids were eluted with 1N ammonium hydroxide. Eluate containing tracer was evaporated to dryness under nitrogen, and the residue taken up in water for subsequent chromatographic measurement of 3-hyp on the

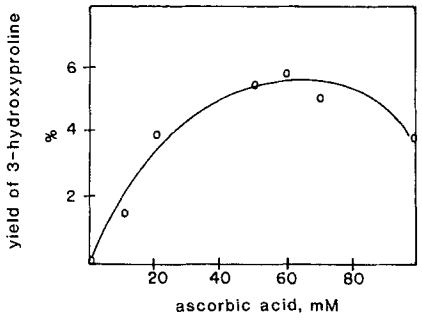

Fig. 1. Effect of ascorbic acid concentration on yield of 3-hyp.

amino acid analyzer. With different ascorbic acid concentrations the yield of 3-hyp varied from 1.5 to almost 6% with optimal yield at approximately 60 mM ascorbic acid (Fig. 1).

Purification of 3-Hyp from Reaction Products

Amino acid analysis of reaction mixtures revealed at least seven compounds with ninhydrin products absorbing strongly at 440 nm and one at 570 nm (Fig. 2). Most of these compounds were eluted before proline and, from their

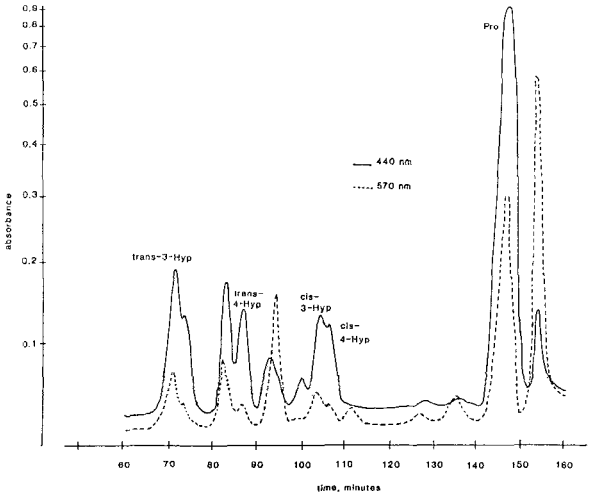

Fig. 2. Analysis of compounds produced in the peroxidation of proline. 3-Hyp and accompying products were chromatographed on the amino acid analyzer with ninhydrin assay of the effluent.

positions, we identified peaks for the trans- and cis- isomers of both 3- and 4-hyp. The identity of the remaining 440 nm and the 570 nm peaks is unknown; it is conceivable that dihydroxyprolines are formed, but we lack reference standards for such compounds. We sought conditions for purification of the trans-3-hyp on a larger scale.

A 2 liter reaction mixture containing 60 mM ascorbic acid and the other components as indicated above was divided equally between two 2 liter flasks. To avoid over-shooting the desired temperature owing to heat generation, the flasks and their contents were cooled to 15°C prior to addition of the peroxide, and then warmed to 37° C. After 10 minutes, tracer 3-hyp was added and the reaction mixture was passed through an AG 50 column (60 x 2.5 cm). The column was washed with 1 liter of water and reaction products were eluted with 1N ammonium hydroxide. Fractions containing tracer were pooled, concentrated to dryness in a flash evaporator, and the residue dissolved in 50 milliliters of water. This solution was decolorized with 5 grams Norit A and filtered. The clear filtrate was evaporated as before and the residue was dissolved in 10 ml water in preparation for chromatography.

The sample was first applied to AG 50 (1 x 16 cm) to separate 3-hyp from unreacted proline by elution with 0.5N HCl. The 3-hyp fractions, identified by the radioactive tracer, were pooled and taken to dryness. An aqueous solution of the residue was chromatographed in small portions on a column of amino acid analyzer resin (50 x 0.9 cm) eluted with 40 milliliters of Durrum's Pico Buffer "A" (18) adjusted to pH 2.93 followed by 45 milliliters of unadjusted Buffer "A". The tracer eluted in several milliliters between 35-45 milliliters of effluent.

Combined fractions from the multiple purifications were desalted on AG 50 by elution with 1N ammonium hydroxide and the residue obtained after evaporation was dissolved in water for investigation on the amino acid analyzer. A single peak for trans-3-hyp was obtained. The overall yield in the hydroxylation reaction after correction for losses during purification was 4.8%.

Additional criteria of purity of the 3-hyp thus formed are as follows. There were no other peaks in the trace from the amino acid analyzer column. In paper electrophoresis the material migrated with the authentic reference standard and there was only a minor (<5%) contaminant, revealed as a purple spot with ninhydrin. The mass

spectrum (Fig. 3) indicated identity with the reference and these spectra agreed with those reported earlier (17). Optical rotation in water gave $(\alpha)_D^{25}$ - -13.55°, in good agreement with the value of -13.7° obtained under these conditions in our laboratory, but lower than the literature (19) value of -19°.

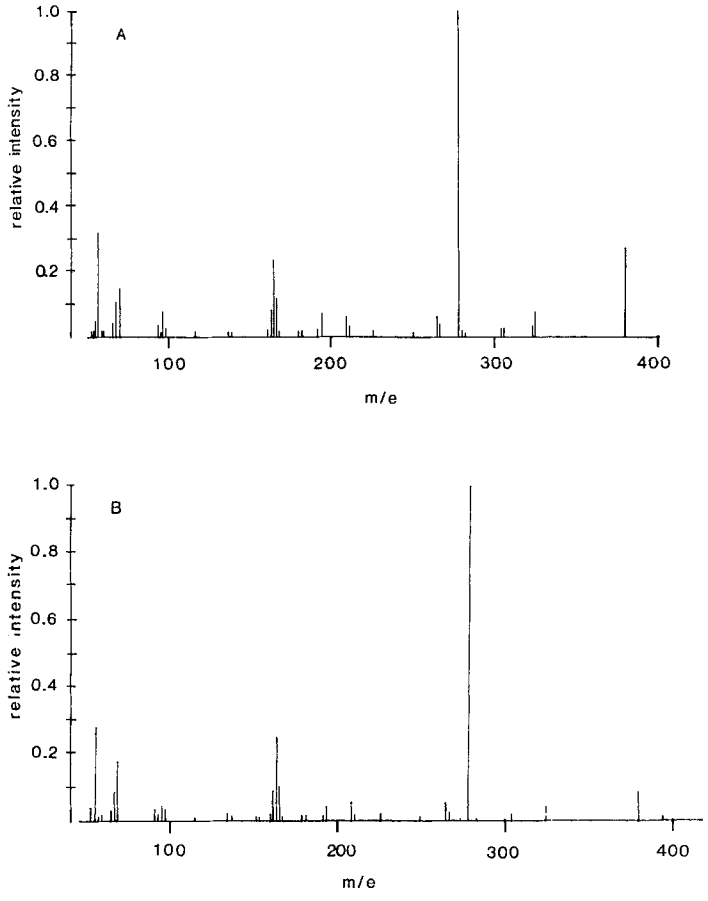

Fig 3. Mass spectra of the butyl ester of trifluoroacetyl-3-hyp. 3-Hyp was produced from proline by peroxidation and purified chromatographically before derivatization. Fig. 3A is the mass spectrum of the experimental material and Fig. 3B that of authentic, derivatized 3-hyp.

Preparation of U-^{14}C-3-Hyp from U-^{14}C-Proline

An 8 milliliter reaction mixture with component concentrations as indicated above, but with only 42 micromoles of ^{14}C-proline (sp. act. 14.6 mCi/mmol) was incubated for 10 minutes at 37° C. The reaction mixture was transferred to a column as AG 50 (16 x 1 cm) and, after washing with 70 milliliters of water, amino acids were eluted with 0.5 N HCl. The radioactivity appeared in the effluent in three peaks (Fig. 4); 17.4% of it was associated with fractions containing 3-hyp, while 2.9% was a minor contaminant that eluted

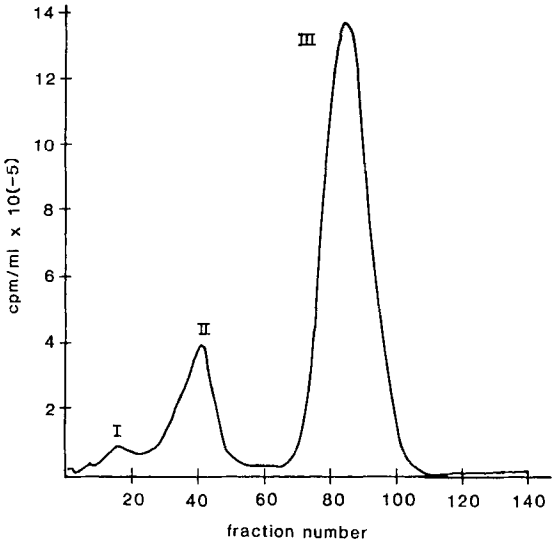

Fig. 4. Initial chromatographic analysis of reaction mixture resulting from peroxidation of ^{14}C-L-proline. Chromatography on AG 50; elution with HCl. Peaks, I, II, III, contain, respectively, a contaminant, 3-hyp, and proline. Fraction volume was 2.0 ml.

before 3-hyp. Seventy-eight percent of the activity was present in the proline peak. The fractions containing 3-Hyp were evaporated and the residue was reconstituted in water; remaining HCl was eliminated by passage through AG 11A8 (52 x 1 cm) and elution with

water. Radioactive fractions were combined, evaporated, and desalted on a small column of AG 50 with 1N ammonium hydroxide elution. This sample, after evaporation and redissolution in 0.01N HCl was chromatographed on amino acid analyzer resin (58 x 0.9 cm), as described above. Several radioactive peaks were present (Fig. 5), the first corresponding to the position of 3-hyp. This material was desalted; an aliquot co-eluted with ^{3}H-3-hyp on the amino acid analyzer. The measured specific activity of the synthesized 3-hyp was 15.3 mCi/mmole, within experimental error of the starting ^{14}C-proline specific activity. Recovery was 2.7% uncorrected for purification losses.

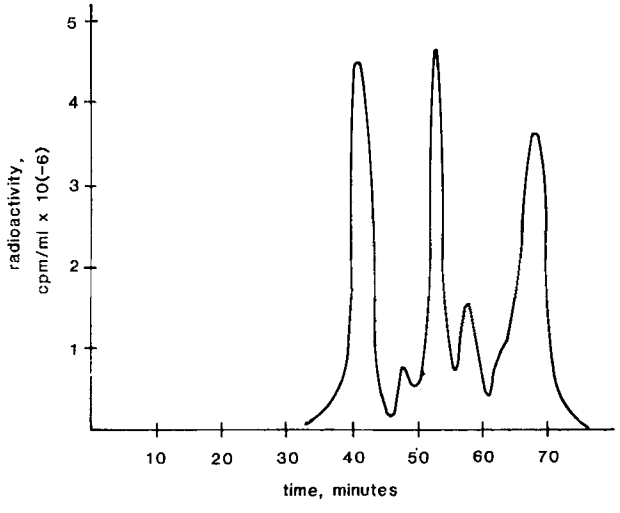

Fig. 5. Chromatography of radioactive 3-hyp produced from proline by peroxidation. Fractions from AG 50 chromatography containing 3-hyp (Peak II, Fig. 4) were chromatographed on amino acid analyzer resin and the effluent assayed for radioactivity. Flow rate was 1.0 ml/min. The peak at 40 minutes corresponds to the elution position of authentic 3-hyp.

DISCUSSION

Hydroxylation of proline in reaction mixtures containing hydrogen peroxide, ferrous ions, and ascorbic acid has been studied in the context of models for in vivo systems (20-22). The earlier studies did not provide definitive data for the formation of rigorously-identified 3-hyp in these reaction mixtures. The present work adds such data and offers a sound method for preparation of labeled 3-hyp from labeled proline.

In our studies the identity of the product as 3-hyp was established by several criteria: ion-exchange chromatography, paper electrophoresis, and mass spectrum. The optical rotation of the product indicates that epimerization of the alpha-carbon does not occur during peroxidation; this is a distinct advantage over other chemical methods which often generate racemic mixtures. The present method permits preparation of 3-hyp of very high specific activity since there is no dilution of the precursor proline, as occurs with the biosynthetic method. Additionally, the unreacted proline can be recovered from the reaction mixture.

We have not been able to confirm the report (23) of a 15% yield of 3-hyp from proline treated with hydrogen peroxide at elevated temperature. In that work, the identity of 3-hyp was inferred from its elution position without reference to a standard, while its quantity was based on color yields for 4-hyp. In our hands the described approach gave yields no greater than 1-2%.

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