A New Method for Isolation of Hydroxy-L-proline and L-Proline from Gelatin

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Several investigators have reported that commercial L-proline is contaminated with small amounts of hydroxy-L-proline (1-3). During the course of investigations in this laboratory all of five commercial samples proved to contain up to 3.5 per cent hydroxyproline when tested by the method of Neuman and Logan (1). The preparation of L-proline from gelatin by the formation of complex salts is tedious and gives relatively low yields (4-8) of the contaminated product. Attempts to prepare L-proline and hydroxy-L-proline by the treatment of gelatin hydrolysates with nitrous acid also gave low yields (9) or impure products (10). Since a simple efficient method for the separation of L-proline and hydroxy-L-proline should make possible their preparation via the nitrosoamines as well as from the impure commercial products, the use of large columns was investigated.

Attempts to modify the method of Rietz et al. (11) from an analytical to a large scale preparative method were unsuccessful. Direct chromatography on cellulose columns as carried out by Radhakrishnan and Meister (2) always resulted in extensive overlaps of the proline and hydroxyproline peaks.

Moore and Stein (12) obtained good separation of proline and hydroxyproline in analytical amounts on a Dowex 50 column with a citrate buffer eluant. Since a more easily removable eluant was desirable for preparative purposes, dilute hydrochloric acid, as recommended earlier by the above authors (13), was used in the present work. Gradient elution, starting with 0.5 N hydrochloric acid in

**EXPERIMENTAL**

**Preparation of Proline and Hydroxyproline**—Gelatin, 500 gm., was added to 3 l. of 6 N HCl in a 5-l. round bottom flask and refluxed for 20 hours, after which the mixture was concentrated to a volume of 2 l. by distillation. The hydrolysate was decolorized by boiling for 10 minutes with 50 gm. of Norit charcoal. The clear yellow filtrate was divided into two equal portions for treatment with nitrous acid. Since a more easily removable eluant was desirable for preparative purposes, dilute hydrochloric acid, as recommended earlier by the above authors (13), was used in the present work. Gradient elution, starting with 0.5 N HCl, was found to reduce the volume of eluant necessary to obtain the two pure amino acids.

![Fig. 1. Separation of proline and hydroxyproline by column chromatography on Dowex 50 resin. The column was 9.5 x 80 cm. Gradient elution was carried out with 0.5 N hydrochloric acid in the 12.5-liter constant-volume mixer and 1 N hydrochloric acid in the reservoir. Concentrations were determined by the method of Pies et al. (15). The identification of the peaks was confirmed by paper chromatography.](http://www.jbc.org/)

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with several 100-ml portions of water. Gradient elution was begun with a constant volume mixer containing 12.5 l. of 0.5 N HCl and an 18-l. reservoir containing 1 N HCl. Hourly fractions, varying from 300 to 400 ml., were collected on a Packard time flow collecting apparatus. To locate the peaks, 0.2 ml aliquots from every third fraction were neutralized with saturated Na2CO3 solution and treated with 3 ml. of 0.15 per cent ninhydrin in glacial acetic acid according to the method of Pies et al. (15). Quantitative determinations by this method were then made on suitable dilutions by measurement of the optical density at 350 nm on a Beckman model DU spectrophotometer, after allowing the reaction to proceed for 2 and 4 hours for hydroxyproline and proline, respectively. The results of the separation are shown in Fig. 1.

Crystallization of Proline—The fractions containing proline hydrochloride were combined and reduced to a syrup under vacuum. Water was added and the mixture was concentrated again. Free proline was regenerated by passing the syrup, diluted with water, through an Amberlite IR-4B column in the free amine form and washing with four volumes of water. The eluate was evaporated to dryness and the dry proline was taken up in 200 ml. of 95 per cent alcohol. The solution was decolorized by boiling with acid-washed Norit charcoal for 5 minutes. Two volumes of ether were added to the filtrate and the mixture was chilled. The proline was filtered off, dissolved in the minimal amount of boiling absolute alcohol and placed in the deep freeze. The solid was re-crystallized by the addition of 70 ml. of ether. A yield of 6 gm. per 100 gm. of gelatin was obtained. The separation occurred in relatively high yields.

DISCUSSION

Hamilton and Ortiz (9) attempted a preparation of proline and hydroxyproline from gelatin by extracting the nitroso derivatives with ether. In this laboratory, after four extractions with ether most of the hydroxyproline derivative remained in the aqueous phase. It was confirmed that extraction with ethyl acetate was more effective; however, the less volatile solvent was more difficult to remove and destruction of proline and hydroxyproline occurred (9).

Removal of HCl on Amberlite IR-4B resin before separation of proline and hydroxyproline and an analysis of the material showed that the imino acids accounted for only two-thirds of the dry HCl-free weight. The impurity was alcohol-soluble. Upon separation on the Dowex 50 column there was an overlap of the impurity with the hydroxyproline peak. This, however, caused no difficulty in the subsequent isolation of pure hydroxyproline.

SUMMARY

A simple method for the preparation of hydroxy-L-proline and L-proline from gelatin is reported. A gelatin hydrolysate is treated with nitric acid to destroy all a-amino acids. L-Proline and hydroxy-L-proline are regenerated by hydrolysis of their nitrosamines with 6 N HCl. After extraction of ether-soluble materials the imino acids are separated on a Dowex 50 column by gradient elution with diute HCl. Hydroxy-L-proline and also L-proline uncontaminated with hydroxy-L-proline are obtained in relatively high yields.

REFERENCES

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