Determination of hydroxyproline in tissue

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Overview

Hydroxyproline is uniquely present in collagen and can be used to determine the amount of collagen present in various tissues. The hydroxyproline content of collagen in a particular tissue must be known, however, in order to use an appropriate conversion factor. The following hydroxyproline procedure is based on the oxidation of the imino acid to pyrrole 2-carboxylic acid which is converted subsequently to pyrrole with heat. Pyrrole can be extracted with toluene and will form a colored chromogen with *p*-dimethylamino benzaldehyde (Ehrlich's reagent).

Reagents

Hydroxyproline standard, 0.1 mg/ml. Dissolve 250 mg of vacuum-dried L-hydroxyproline (Sigma, Cat. No. H-6002) in 25 ml 0.001 N HCl. One ml of this solution is diluted to 100 ml with 0.001 N HCl.

Hydroxyproline working standard, 10 μg/ml. Dilute 10 ml 0.1 mg/ml hydroxyproline standard with deionized water to 100 ml.

Potassium borate buffer, pH 8.7. Mix 61.84 g boric acid and 225 g KCl in about 800 ml deionized water. Adjust the pH to 8.7 with 10 N and 1 N KOH and make the final volume up to 1 liter. Prepare a 1:5 dilution of buffer as needed.

Chloramine T solution. Prepare fresh daily a solution of 536.4 mg of chloramine T (Sigma, Cat. No. C-9887) in 10 ml methyl cellosolve (ethylene glycol monomethyl ether, Fisher, Cat. No. E-182).

Sodium thiosulfate, 3.6 M. Dissolve 893.4 g sodium thiosulfate in about 900 ml deionized water and bring the final volume to 1 liter. Store under toluene at room temperature for several weeks.

Ehrlich's reagent. Add 27.4 ml concentrated sulfuric acid to 200 ml absolute ethanol in a beaker and cool the mixture. In another beaker, place 120 g *p*-dimethylaminobenzaldehyde (Fisher, Cat. No. D71-100) and 200 ml absolute ethanol and then add slowly with stirring the acid-ethanol mixture from the first beaker. The solution can be stored in the refrigerator for several weeks and the crystals that form can be redissolved by warming the solution.

Glass culture tubes, 150×16 mm, screw-capped with Teflon liners (Fisher, Cat. No. 14-930-10E) are used both for hydrolysis and for hydroxyproline oxidation.

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Procedure

Tissue hydrolysis

1. Place 25–350 mg wet weight of tissue in dry culture tube of known weight.
2. Dry the samples in an oven at 65° C for 18–24 hours and then allow the tubes to cool to room temperature in a desiccator.
3. Weigh the tubes.
4. Add 2.0 ml 6 N HCl, cap and hydrolyze the samples at 110° C for 24 hr.
5. Evaporate the samples to dryness with a stream of nitrogen.
6. Add 10.0 ml deionized water to each tube and mix well.

Hydroxyproline determination

1. Transfer 0.2 ml hydrolyzate to a clean, labeled culture tube followed by 1.6 ml deionized water.
2. Prepare a set of tubes containing known amounts of hydroxyproline (1.0–8.0 μg) and water as reagent blank.
3. Add 1.0 ml 1:5 diluted borate buffer to all tubes.
4. Add 0.3 ml chloramine T to each tube in a timed sequence to oxidize the hydroxyproline and mix well.
5. After 20 min, add 1.0 ml sodium thiosulfate and mix well.
6. Add about 1.5 g potassium chloride to saturate all tubes. If indole, dehydroproline, or similar compounds are anticipated in the samples, extract with 2.5 ml toluene and discard the toluene extract.
7. Cap and heat the tubes in boiling water for 20 min.
8. Cool tubes to room temperature, add 2.5 ml toluene, and cap all tubes tightly. Invert the tubes 100 times or shake them about 5 min.
9. Centrifuge the tubes at low speed briefly and transfer 1.0 ml toluene extract to labeled 12 × 75 mm test tubes.
10. Add 0.4 ml Ehrlich's reagent and allow the color to develop by 30 min.
11. Read the absorbances at 565 nm against a reagent blank.
12. A linear regression of absorbance versus μg hydroxyproline standard can be used to calculate the hydroxyproline content of each unknown sample. Multiply the values by the dilution factor of 50 to determine the hydroxyproline content of the original hydrolyzate.

Special considerations

1. The toluene extract in step 6 of hydroxyproline determination can be used to determine the concentration of proline by a periodate oxidation method.[1]
2. The potassium chloride can be added at any point before oxidation with chloramine T without any effect on the color yield.
3. Toluene may be added before heating the samples in order to save time in removing caps and recapping tubes in step 8. Any toluene lost appears to be proportional in all tubes, but the caps need to be checked for tightness since they frequently become loose on cooling after heating.
4. Centrifugation in step 9 may not be necessary if no emulsion is present in any of the samples.
5. Hydroxyproline can be determined in 48–60 samples in about 4 hours.

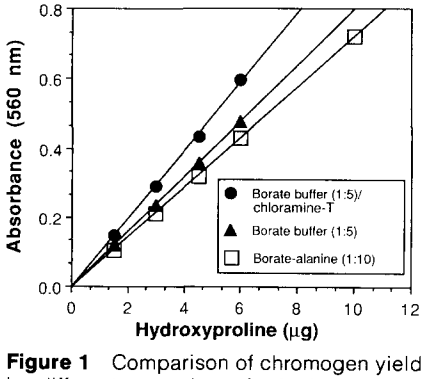

Figure 1 Comparison of chromogen yield by different procedures for hydroxyproline determination. Open squares represent Blumenkrantz and Asboe-Hansen's procedure[1] that uses 0.1 M borate + 1% alanine buffer; closed triangles represent the use of 0.2 M borate buffer only; and the closed circles represent the use of 0.2 M borate buffer + 50% less chloramine T for oxidation and 50% less sodium thiosulfate.

Discussion

The present procedure involves several modifications of earlier procedures.[1-5] Instead of a dilute borate buffer used here, earlier procedures used either a 0.2 M sodium pyrophosphate buffer, a 1.0 M borate buffer, or a combined 1.0 M borate–10% alanine buffer. All of these buffers gave significantly less chromogen.[1] The hydroxyproline standards (*Figure 1*) gave a higher color yield with Ehrlich's reagent if oxidation was performed with 1:5 diluted (0.2 M) borate buffer only than with 1:10 diluted 0.1M borate–1% alanine buffer. The best color yield was achieved when 50%

less chloramine T was used in the oxidation step and 50% less sodium thiosulfate was used to stop oxidation as compared to that of Blumenkrantz and Asboe-Hansen.[1]

The need to adjust the pH of the hydrolyzates as described in other procedures[2-5] has not been observed in our laboratory. This has not been a problem for us when appropriate aliquots of hydrolyzates are taken to keep the concentration of hydroxyproline within the range of the assay.

Our results indicate the modification of reagents and amounts produced a very sensitive assay of hydroxyproline, which can be applied to a large number of samples in a relatively short time period. Furthermore, the laborious step of adjusting pH was eliminated and the number of steps has been kept to a minimum.

References

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