Hydroxyproline Assay Using NaBr/NaOCl

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[Abstract] Hydroxyproline (Hyp) is a major constituent of a relatively few proteins that are major structural components of the extracellular matrix and primary cell wall of animals and plants respectively. Significant amounts of the cyclic amino acids proline and hydroxyproline decrease polypeptide flexibility; thus proline/hydroxyproline-rich proteins are ideal scaffold components. Collagens typify animal tissues but extensins, arabinogalactan proteins (AGPs) and their close relatives, collectively referred to as hydroxyproline-rich glycoproteins (HRGPs), typify plants (Lamport *et al*., 2011). While collagens are minimally glycosylated generally via a galactosyl hydroxylysine linkage, plant HRGP glycosylation involves short neutral oligosaccharides (in extensins) or much larger acidic polysaccharide substituents (in AGPs) O-linked via the hydroxyproline hydroxyl group. Hydroxyproline assay is thus an integral part of their characterization and dominates the biochemical properties of these glycoproteins. The colourimetric assay described here quantifies ***free*** hydroxyproline (*e.g.* released by acid hydrolysis) based on Kivirikko and Liesmma (1959) with hypobromite as an oxidant but modified by avoiding the use of hazardous liquid bromine. A number of oxidants have been used over the years, Vogel (1961, page 395) explains the preference for hypobromite as follows: *"Hypochlorites tend to react slowly with reducing agents. Hypobromites although rather unstable when prepared directly from bromine and alkali, often react more rapidly; it is therefore advantageous to produce hypobromite in situ by adding an excess of bromide to the sample of hypochlorite:"* $OCl^- + Br^- \rightarrow OBr^- + Cl^-$ *"By this means the relative stability of hypochlorite is combined with the more effective oxidizing properties of hypobromite."*

Materials and Reagents

1. NaOCl (Lab bleach)
2. NaOH
3. NaBr
4. 6 N HCl
5. Dilute hypobromite
6. p.dimethylaminobenzaldehyde (Sigma-Aldrich, catalog number: 156477)
7. n.propanol (Sigma-Aldrich, catalog number: 402893)

Equipment

1. 2 ml screw-cap microtube (SARSTEDT AG)
2. Microplate reader or spectrophotometer

Procedure

1. Prepare dilute sodium hypobromite (NaOBr) from NaOCl, mix equal volumes (a) + (b) (*e.g.* 5 ml each) (prepare fresh weekly-store at 4 °C).
   a. Add 775 µl lab bleach to 10 ml 4% NaOH (fresh weekly).
   b. Prepare 100 mM NaBr (1.03 g in 100 ml 4% NaOH) (stable).
2. Add 250 µl aqueous sample to 2 ml screw-cap microtubes (*e.g.* SARSTEDT AG).
3. Add 500 µl dilute hypobromite to:
   a. Analysis samples each in 250 µl distilled water.
   b. Hyp standards of 2.5, 5.0, 7.5 and 10 µg, each in 250 µl distilled water.
   c. A reagent blank contains reagents plus an additional 250 µl distilled water.
4. Mix and leave for 5 min to oxidize at room temperature.
5. Add 250 µl 6 N HCl.
6. Add 500 µl 5% p.dimethylaminobenzaldehyde in n.propanol (total volume = 1.5 ml).
7. Mix and heat at 70 °C for 15 min, then cool in ice-water.
8. Measure absorbancy of samples and standards at 560 nm against the reagent blank. *e.g.* 10 µg Hyp → ~680 mAUs
9. Construct the standard curve and calculate sample values by interpolation.

Recipes

1. This assay determines *free* hydroxyproline, best prepared by hydrolysis peptide bonds in the sample to be analysed (6 N HCl at 110 °C for 18 h) followed by removal of HCl in vacuo and redissolving the hydrolysate in distilled water. Interestingly *free* O-glycosylated Hyp can be assayed directly.
2. Use aqueous Hyp standards over a 2.5 to 10 µg range. It is convenient to prepare a Hyp standard of 100 µg/ml in distilled water stored frozen; mix well after thawing! The reagent blank contains all the reagents with distilled water as a substitute for the sample.
3. 5% w/w p.dimethylaminobenzaldehyde in *n*-propanol.

Acknowledgments

This protocol is adapted from Kivirikko and Liesmaa (1959) and Lamport *et al*. (2012).

References

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