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MODIFIED ASSAY FOR DETERMINATION OF HYDROXYPROLINE IN A TISSUE HYDROLYZATE

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Summary

A modified assay for the determination of hydroxyproline in tissue is presented. The modifications greatly reduce the time required for analysis of excised tissue as first introduced by Stegemann and Stalder [1]. These modifications include a change in the technique for tissue hydrolysis and a change in the preparation of the hydroxyproline oxidizing agent. The analysis utilizes the standard addition technique, eliminating the need for correction of matrix effects between the specimen and standard. This paper attempts to give a complete detailed description of the assay such that the procedure may be repeated without requiring additional reference material.

Introduction

Collagen is one of the more important structural proteins in the body being of particular importance in connective tissues by providing their durability. As such, knowledge of at least the amount of collagen in a particular tissue is essential for the complete understanding of the structural and mechanical properties of that tissue. In particular, Fields and Dunn [2] have suggested that it is the structural proteins, collagen primarily, which are responsible for the tissues' echographic visualizability. O'Brien [3] has shown that, to a first approximation, the tissue's collagen content can be mathematically related to the ultrasonic velocity and attenuation. Pohlhammer and O'Brien [4] have shown that the ultrasonic scattering can also be mathematically related to the collagen content, scattering defined here as the difference between attenuation and absorption of the ultrasonic wave.

Collagen is one of the few proteins which contains the amino acid hydroxyproline. In fact, most of the hydroxyproline in vertebrates is found in this pro-

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TABLE I

EXAMINATION OF ERROR IN DETERMINING COLLAGEN CONTENT BY ASSUMING THAT THE ELASTIN CONTENT IS NEGLIGIBLE

Tissue *	Measured collagen content **	Measured elastin content **	Calculated hydroxy-proline **	Calculated collagen **	% error ***
Liver	0.64	0.3	0.086	0.69	7.8
Kidney	1.8	0.5	0.24	1.9	5.6
Spleen	3.5	0.5	0.45	3.6	2.9
Skin	67.6	0.6	8.5	67.7	0.15
Achilles tendon	86.0	3.0	10.8	86.4	0.47
Aorta	37.0	47.0	5.6	44.5	20.3

* All tissue is rat except Achilles tendon which is human.

** g protein or hydroxyproline per 100 g dry tissue.

*** $\%\ \text{Error} = \frac{\text{calculated collagen} - \text{measured collagen}}{\text{measured collagen}} \times 100.$

tein [5]. For example, collagen extracted from skin (a tissue with a high collagen content) has been shown to contain from 10—14 g hydroxyproline/100 g protein for various animal species [6—11]. Elastin, another of the connective tissue proteins, contains small quantities (1—3 g/100 g protein) of hydroxyproline [9,11], thus representing the major source of error for determining collagen content by a hydroxyproline analysis. Table I compares published [12] values of collagen and elastin content with calculated values of collagen content assuming that the elastin content is negligible for various tissues. The total hydroxyproline content is calculated by summing the contribution due to collagen and that due to elastin, assuming average values of 12.5 g and 2.0 g hydroxyproline/100 g protein for collagen and elastin, respectively. Collagen content was then calculated based on this value for hydroxyproline, but assuming that all of it was due to collagen. It can be seen that this is a reasonable assumption for tissue in the high and medium collagen content range given the precision and accuracy of the assay. However, for tissue such as aorta which is predominantly elastin, the error is substantial.

For tissue with relatively high elastin content with respect to collagen content, extraction procedures are available for separating and purifying the collagen [13—15]. However, for most tissues, it is possible to obtain a reasonable first approximation of collagen content by utilizing a biochemical assay for hydroxyproline and assuming the error due to elastin is negligible.

The method described herein is a modification of an assay first introduced by Stegemann and Stalder [1]. The modifications suggested reduce the time involved in the determination of tissue collagen content while eliminating possible matrix effects of the tissue hydrolyzate with no loss of sensitivity. These modifications include a change in the hydrolysis procedure and incorporation of the standard addition technique into the assay procedure.

Materials

Chloramine-T reagent (0.050 mol/l)

1.41 g chloramine-T (Eastman) is dissolved and brought to 100 ml with distilled water.

This reagent lacks stability and is prepared immediately before use.

Aldehyde-perchloric acid reagent

15 g *p*-dimethyl-amino-benzaldehyde (Eastman);
60 ml *n*-propanol (Mallinckrodt);
26 ml perchloric acid (70%, reagent grade) (Mallinckrodt).
Bring to a volume of 100 ml with distilled water.

This reagent is stable for about 1 h and is therefore prepared shortly before use.

Buffer solution

133 g citric acid · 1 H_2O (Mallinckrodt);
32 ml glacial acetic acid (Mallinckrodt);
320 g sodium acetate · 3 H_2O (Mallinckrodt);
91 g sodium hydroxide;
800 ml *n*-propanol.
Bring to a volume of 3000 ml with distilled water.
Adjust pH between 6.0—6.5 with 0.2 mol/l NaOH solution.
Bring to a volume of 4000 ml with distilled water.
1—2 ml of toluene are added as a preservative.

This reagent will remain stable for about 2 months if refrigerated in a dark bottle.

Hydroxyproline standard

Primary Standard — 0.5 g stock hydroxyproline (Calbiochem) is dissolved in 1000 ml of the buffer (500 μg hydroxyproline per ml buffer).

Secondary — 20 ml of primary standard is brought to a final volume of 100 ml with the buffer (100 μg/ml).

4 μg/ml 4 ml of secondary to a final volume of 100 ml;
2 μg/ml 2 ml of secondary to a final volume of 100 ml;
0 μg/ml pure buffer.

Procedure

A flow chart of the procedure is shown here. A tissue sample of known weight is placed in the homogenizer (A. Thomas Co., Philadelphia, PA, U.S.A.). The tissue may be freshly excised or dried. Hydrochloric acid is added in a ratio of 10 mg tissue (wet weight) to 1 ml of 6 mol/l HCl. Since fresh tissue is not required for the assay, the specimen may have been dried by a freeze drying method or by placing the tissue in a desiccator under vacuum. Sufficient tissue is homogenized such that at least four samples may be obtained from the homogenate suspension. 2-ml aliquots of the homogenate are placed in four Pyrex test tubes for hydrolysis. In this way each of the four samples is identical in tissue content.

A glass ball is placed over the opening of the tubes to prevent loss from boiling and the tubes are placed in a pressure vessel (National Presto Industries) containing about 2 cm of water and heated to 120°C at an absolute pressure of about 210 kPa (1 Pa = 1 N/m^2) under a hood. Hydrolysis is complete within 2—4 h.

FLOW CHART OF THE MODIFIED HYDROXYPROLINE ASSAY PROCEDURE

1. Tissue is excised and weighed.
2. Tissue may be dried and reweighed to determine water content.
3. Hydrolysis: 10 mg tissue/1 ml 6 mol/l HCl. Heat at 120°C in a pressure vessel for 2—4 h.
4. Samples dried overnight in a vacuum desiccator.
5. Buffer is added to dilute samples to assay sensitivity range. Standards are prepared.
6. Standards are added to samples.
7. Chloramine-T reagent is prepared. 1 ml is added to each tube and allowed to react for about 20 min. During reaction period, the aldehyde-perchloric acid reagent is prepared.
8. 1 ml of aldehyde-perchloric acid reagent is added to each sample. Samples are placed in hot water bath (60° C) and allowed to react for 15 min.
9. Samples are cooled, absorbance is read at 550 nm for each sample and standard. Sample concentrations are determined from the standard addition curve.

Upon completion of hydrolysis, the tubes are placed in a vacuum desiccator with the glass balls removed. The sample tubes are then heated to about 50—60°C while a vacuum is drawn. The vacuum should not be so strong that the samples boil violently. A cold trap (acetone and dry ice) and a sodium hydroxide trap (solid NaOH in a vacuum chamber) are placed along the vacuum line to prevent moisture and HCl from damaging the vacuum pump. The tubes are left in the desiccator until most of the HCl has been removed and the samples are dry. Drying is usually completed overnight, depending upon vacuum strength.

Any remaining HCl is neutralized upon addition of the buffer. The samples are brought to a concentration of about 0.1—4.0 μg hydroxyproline per ml buffer solution. Equal buffer volume is added to each of the four equivalent samples. For most tissue, this means adding 2—4 ml of buffer to bring the sample to the proper concentration. However, for certain high collagen content tissues, such as skin and tendon, greater dilution will be required. The exact dilution is empirically determined. A 2-ml volume is removed from each sample tube and transferred to clean tubes for hydroxyproline determination.

The standard hydroxyproline solutions, prepared beforehand, are now added to the four equivalent 2-ml samples just obtained. As a specific example, to the first sample, 1 ml of 0 μg/ml standard is added, to the second, 1 ml of 1 μg/ml, to the third, 1 ml of 2 μg/ml and to the fourth 1 ml of 4 μg/ml. A blank, consisting of 3 ml buffer is also prepared and treated identically to the samples, providing the spectrophotometer's baseline reading.

Only the volume of chloramine-T reagent required is now prepared, due to its instability. One and a half ml of the 0.05 mol/l chloramine-T solution is added to each of the tubes and allowed to react for 20—25 min. During the reaction period, the aldehyde-perchloric acid reagent is prepared, and then 1.5 ml of it is added to each of the tubes after the chloramine-T reaction period. The tubes are then placed in a 60°C water bath for 15 min during which time the red chromophore develops. The samples are cooled to room temperature either by allowing them to sit or by placing them in an ice water bath for a short period of time. Absorbance is read within 3 h at 550 nm or the red chromophore starts to break down and absorbance decreases.

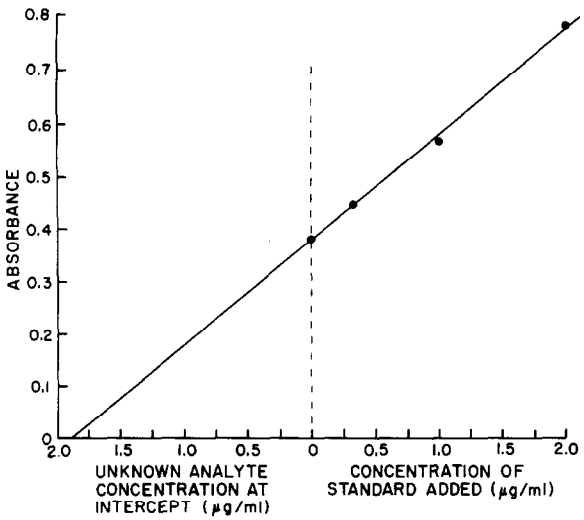

Fig. 1. Standard addition curve for determination of the hydroxyproline concentration of a tissue hydrolyzate. This particular tissue sample is a purified bovine tendon collagen (Calbiochem) which requires serial dilutions in order for the hydroxyproline concentration to be within the assay sensitivity range. These dilutions must be taken into account to determine the actual tissue hydroxyproline concentration.

A plot is made of absorbance vs. concentration of hydroxyproline standard in solution as shown in Fig. 1. The equation of the line is determined by performing a least squares linear regression analysis on the data points. For the collagen sample in Fig. 1 the equation of the line is

$$\text{Absorbance} = 0.2 \times [\text{hydroxyproline}] + 0.38 \quad (1)$$

The point at which the absorbance is equal to zero is the concentration of hydroxyproline in solution due to the tissue hydrolyzate. After correcting for all dilutions made of the tissue hydrolyzate, the hydroxyproline content of the tissue may be determined.

Results and Discussion

Vacuum sealed Pyrex tubes were initially used for the hydrolysis procedure, to prevent oxidation and loss of the sample. The procedure employed a temperature around 110°C and required 15 to 20 h for complete hydrolysis. This proved to be both difficult and time consuming when a large number of samples were to be analyzed. Since at higher temperatures, the hydrolysis time can be reduced [16], a standard lab pressure vessel was employed to obtain temperatures around 120°C for hydrolysis without the need for sealed tubes, the latter because the water surrounding the hydrolysis tubes boils at a lower temperature than the HCl-tissue mixture.

The standard addition technique is commonly incorporated into analytical assays for correction of matrix effects. The matrix is defined as the solvent plus anything else in the solution being analyzed besides the analyte. In the case of a tissue hydrolyzate, it is impossible to synthesize exactly the matrix that the

hydroxyproline is found in. This means that, if the standards are separate from the samples, there is a possibility of error due to the difference between the matrixes of the sample and standard. Incorporation of standard into the sample eliminates this error.

The original assay [1] required that the chloramine-T be dissolved in the buffer solution diluted with water and *n*-propanol. It was found that the chloramine-T failed to stay in solution with this particular preparation. To avoid this problem, the solvent was changed to water and the solution was prepared just prior to use. The change in solvent did not affect the accuracy or sensitivity of the assay. Others [17] have satisfactorily used water as the solvent for the chloramine-T.

The effect of minor concentration variations of the two reagents on the assay repeatability was determined. No change was made in the buffer. The chloramine-T reagent was modified by dissolving 1.41 g of chloramine-T in distilled water and bringing it to a volume of either 95 ml or 105 ml, that is 0.053 mol/l or 0.048 mol/l, respectively. The aldehyde-perchloric acid reagent was modified by using 55 ml or 65 ml of *n*-propanol and bringing it to a volume of 95 or 105 ml, respectively, thus resulting in a 1.06 mol/l or 0.96 mol/l *p*-dimethyl-aminobenzaldehyde solution. A sample of known hydroxyproline concentration was analyzed by the above procedure, using either both reagents with the lower molarities or both reagents with the higher molarities. In each of the two cases, there was no significant difference in the amount of hydroxyproline in the sample as determined by the assay.

Reaction time of the chloramine-T reagent was also varied. It was found that a minimum of 20 to 25 min insured the best color formation of the chromophore but that longer periods of time had a negligible effect on the final assay results.

Two devices were especially useful for handling a large number of samples. The Oxford Pipettor Reagent Bottle is used to add appropriate aliquots of the buffer to samples. It can be set to a specific volume (1—10 ml) and delivers that volume with a precision of 0.5%. The Cornwall Continuous Pipette is used for adding the chloramine-T reagent and the aldehyde-perchloric acid reagent. It has a reproducibility of 0.3% for any number of samples being analyzed.

As the buffer ages, the sensitivity tends to decrease somewhat; so a typical practice is to replace the buffer every two months. Sensitivity of the assay seems to be dependent on the buffer's age more than any other aspect of the assay.

Reproducibility of the assay was demonstrated by analyzing 12 identical samples of a gelatin (Baker) solution with a concentration of 41.2 μg gelatin/ml of 6 mol/l HCl. The samples were found to have hydroxyproline concentrations ranging from 4.2—5.3 μg/ml with a mean value of 4.8 μg/ml and a standard deviation of 0.35 μg/ml.

Table II gives the results of an analysis of different tissues from LAF/J (Jackson Labs, Bar Harbor, ME, U.S.A.) mice. The results are an average of three different samples of each tissue type. The results given are percent hydroxyproline/wet weight of the tissue. The collagen values were calculated assuming 12.5% of collagen is hydroxyproline and elastin content was negligible.

Three identical samples of a stock collagen (Nutritional Biochem) were sent

TABLE II

HYDROXYPROLINE CONTENT DETERMINED BY MODIFIED PROCEDURE AND THE CALCULATED COLLAGEN CONTENT FOR VARIOUS MOUSE TISSUES

Tissue	g Hydroxyproline/100 g tissue	S.D.	g collagen/100 g tissue
Liver	0.032	0.002	0.26
Kidney	0.046	0.004	0.37
Spleen	0.091	0.001	0.73
Tail tendon	7.1	0.003	56.5

to an independent laboratory (Arro Labs, Joliet, IL, U.S.A.) for analysis of hydroxyproline content. Another four samples were analyzed using the procedure described here. The results obtained by this procedure were within the 7% range of values determined by Arro Labs.

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References

1 Stegemann, H. and Stalder, K. (1967) Clin. Chim. Acta 18, 267
2 Fields, S. and Dunn, F. (1973) J. Acoust. Soc. Am. 54, 809
3 O'Brien, W.D. Jr. (1977) in Proc. Ultrasonics International 77, p. 194, Guildford, U.K.
4 Pohlhammer, J.D. and O'Brien, W.D. Jr. (1979) J. Acoust. Soc. Am. 65, S1, S3
5 Miller, E.J. (1975) Chemistry, Structure and Function of Collagen, in Biological Basis of Wound Healing (Menako, L., ed.), p. 243, New York
6 Eastoe, J.E. (1955) Biochem. J. 65, 363
7 Gross, J. and Piez, K.A. (1960) in Calcification in Biological Systems (Sognnaes, R.F., ed.), p. 395, Washington, DC
8 Jackson, D.S., Leach, A.A. and Jacobs, S. (1958) Biochim. Biophys. Acta 27, 418
9 Neuman, R.E. and Logan, M.A. (1950) J. Biol. Chem. 184, 299
10 Fleischmajer, R. and Fishman, L. (1965) Nature 205, 264
11 Miller, E.J., Martin, G.R. and Piez, K.A. (1964) Biochem. Biophys. Res. Commun. 17, 284
12 Chvapil, M. (1967) in Physiology of Connective Tissue, pp. 62, 67, Prague, Czechoslovakia
13 Highberger, J.H., Gross, J. and Schmitt, F.O. (1951) Proc. Natl. Acad. Sci. U.S.A. 39, 286
14 Gallop, P.M. (1955) Arch. Biochem. Biophys. 54, 486
15 Veis, A. and Anosey, J. (1965) J. Biol. Chem. 240, 3899
16 Pashley, D., Claycomb, C.K. and Summers, G.W. (1966) Anal. Biochem. 15, 144
17 Juva, K. and Prockop, D. (1966) Anal. Biochem. 15, 77