A Simple Rapid Method To Estimate Hyaluronic Acid Concentrations in Rooster Comb and Wattle Using Cellulose Acetate Electrophoresis

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A simple rapid method of quantitative analysis of hyaluronic acid (HA) from comb and wattle tissues is described. The technique involves short-time acetone drying and proteolysis of tissues, cellulose acetate electrophoresis of the digests without deproteinization, and measurement of absorbance of the solution of Alcian Blue extracted from the HA band. With this method, the determination of HA, which may take 1 week or longer with other methods, could be made within a day.

Keywords: *Hyaluronic acid determination; comb; wattle; rooster; cellulose acetate electrophoresis*

INTRODUCTION

Comb and wattle tissues contain hyaluronic acid (HA), a nonsulfated glycosaminoglycan (GAG), as their major constituent carbohydrate with small amounts of sulfated GAGs (Schiller and Dorfman, 1956; Swann, 1968; Ng Kwai Hang and Anastassiadis, 1980; Nakano and Sim, 1989). Hyaluronic acid is composed of D-glucuronosyl-*N*-acetylglucosamine repeating disaccharide units with a molecular mass in the range of 10^5–10^7 Da (Muir and Hardingham, 1975). The physiological function of HA in the comb and wattle is not well understood. Its high water holding capacity (Toole, 1982) may be responsible for maintaining the hydration of these tissues. The rooster comb, however, has been known to be an important source to extract HA, which has a wide range of applications [e.g., ophthalmic surgery, orthopedics, and cosmetics (Balazs, 1979; Van Brunt, 1986)].

The hyaluronic acid fraction has been isolated from the comb and wattle and analyzed (Ng Kwai Hang and Anastassiadis, 1980; Nakano and Sim, 1991), but there are few studies that describe quantitative analysis of HA in these tissues. Studies of HA in other tissues suggest that there is no simple, reliable method to estimate the content of this GAG. Such a method is needed for the physiological studies of comb and wattle as well as the preparation of HA from these tissues. Quantitative analysis of HA requires liberation of the total GAG from tissue by proteolysis followed by isolation and quantitative determination of HA. It is important to remove as much non-GAG materials as possible since these materials may contribute to erroneous results. Thus, delipidation of tissues and deproteinization of their proteolytic digest with trichloroacetic acid are common procedures used for analysis of GAG from various tissues (Rodén *et al.*, 1972; Taniguchi, 1982). However, these procedures may not be necessary depending on tissue types and constituents to be analyzed.

Hyaluronic acid can be separated from other GAGs by several methods including ion-exchange chromatography, fractionation with quaternary ammonium salts, and cellulose acetate electrophoresis. Among these, cellulose acetate electrophoresis provides the most rapid and sensitive separation (Taniguchi, 1982). This paper describes a relatively simple method of quantitative analysis of HA from comb and wattle tissues using papain digestion and cellulose acetate electrophoresis.

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MATERIALS AND METHODS

Samples of comb and wattle, which were collected from four 52-week-old Single Comb White Leghorn roosters and stored at −20 °C, were thawed at 4 °C overnight. Each of the comb and wattle samples was cut separately into small 1 mm thick strips and mixed thoroughly. Portions (approximately 300 mg) of each sample were dehydrated at room temperature with three changes of acetone (3 mL every 10 min) with occasional mixing. Acetone was then discarded, and tissues were dried at 80 °C until a constant tissue weight was obtained (about 15 min). The difference in the weight between wet and dry tissue was recorded to calculate the portion of dry matter. Dry tissues (30 mg) were digested with twice-crystallized papain (Sigma) (4 μg/mg of tissue) in 1 mL of 0.1 M sodium phosphate buffer containing 0.005 M EDTA, 0.005 M cysteine hydrochloride, and 0.02% sodium azide having a pH of 6.5 (Scott, 1960). Digestion was carried out at 65 °C for 4 h. After proteolysis, half of each digest was deproteinized by adding cold (4 °C) trichloroacetic acid to a final concentration of 7% (w/v), and the mixture was maintained at 4 °C overnight. The precipitate formed was removed by centrifugation, and the supernatant was transferred to dialysis tubing (molecular mass cutoff, 6000–8000 Da) and dialyzed in running tap water for 24 h and then in cold distilled water for another 24 h.

Papain digests with and without deproteinization and standard HA from human umbilical cord (a gift from Dr. M. B. Mathews, University of Chicago) were then applied to cellulose acetate strips (Sepraphore III, 2.5 × 15.2 cm, Gelman Sciences), which were soaked in pyridine–acetic acid–water (1:9:115), pH 3.5 (Habuchi *et al.*, 1973) for more than 30 min. Papain digests were diluted two to eight times with water to obtain appropriate concentrations before they were electrophoresed. Eight microliters of each sample was applied to one strip to form a 2 cm long band. Electrophoresis was carried out in pyridine–acetic acid–water (see above) for 1 h at 0.5 mA/cm. Following electrophoresis, the cellulose acetate was stained for 3 min in 0.1% Alcian Blue 8GX in 0.1% acetic acid containing 0.02% sodium azide to locate the individual GAGs. Cellulose acetate was washed in three changes of 0.1% acetic acid. The identity of HA band from the papain digest was checked by comparing its electrophoretic mobility to that of standard HA and by its susceptibility to *Streptomyces* hyaluronidase. The digestion with *Streptomyces* hyaluronidase was carried out as described previously (Nakano and Sim, 1989). The amount of HA was estimated following the procedure as

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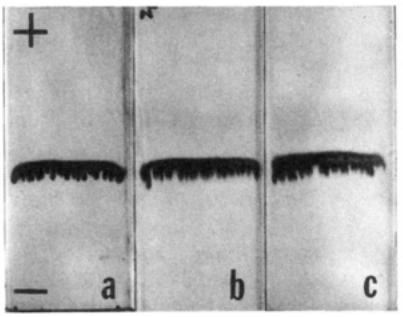

Figure 1. Representative cellulose acetate electrophoretograms of standard HA and the papain digests of comb and wattle tissues: (a) standard HA containing 3 μg of uronic acid; (b) comb papain digest without deproteinization (diluted eight times); (c) wattle papain digest without deproteinization (diluted two times). Glycosaminoglycans migrated toward the anodic end. Slow moving dark band and fast moving faint band contained HA and sulfated GAG, respectively.

Table 1. Hyaluronic Acid Concentrations in Rooster Comb and Wattle Tissues

sample and treatment	hyaluronic acid,[a] μg of uronic acid/mg	
	comb	wattle
dry tissue without deproteinization	44.1 ± 2.8	19.1 ± 2.0
dry tissue with deproteinization	39.8 ± 1.8	17.9 ± 1.7

[a] The HA concentration in the comb is significantly different from that in the wattle ($P < 0.01$). Mean ± SD.

described by Hata and Nagai (1973). The HA band and the adjacent cellulose acetate (as background) were cut with scissors, placed separately in test tubes containing 1 mL of 5% cetylpyridinium chloride (Sigma), while they were damp, and heated in a boiling water bath for 15 min to extract the dye. Absorbance of the dye solution was read at 615 nm. The content of HA was calculated by referring to the standard curve prepared by using known amounts of HA measured as uronic acid (Blumenkrantz and Asboe-Hansen, 1973). A paired *t*-test (Choi, 1978) was used to detect significant differences between means of results.

RESULTS AND DISCUSSION

The electrophoretic bands of standard HA and representative papain digests of comb and wattle tissues are shown in Figure 1. All papain digests had two bands including those of HA and sulfated GAG as shown in our previous study (Nakano and Sim, 1989). The slow moving HA band was completely digested (data not shown) with *Streptomyces* hyaluronidase, an enzyme specific to HA (Ohya and Kaneko, 1970).

There was a linear relationship between the amount of HA and the absorbance in the concentration range from 1 to 6 μg of uronic acid. The HA concentrations determined with and without deproteinization of their papain digests were similar in both the comb and wattle tissues (Table 1). These values were comparable with those estimated from the total uronic acid contents previously determined in the comb (45 ± 6 μg/mg) and wattle (24 ± 3 μg/mg) from roosters of a similar age (Nakano and Sim, 1989), assuming that approximately 93% of total uronic acid is present in HA (Nakano and Sim, 1991). The HA concentrations were about 2-fold higher ($P < 0.01$) in the comb than in the wattle, as was noted previously (Nakano and Sim, 1991).

Hyaluronic acid concentrations were determined in 10 identical samples of comb and wattle without deproteinization. The mean and standard deviation were 46.1 ± 1.8 μg of uronic acid/mg and the coefficient of variation (CV) was 3.9% in the comb. Corresponding values for the wattle were 18.7 ± 1.0 μg of uronic acid/mg and 5.3%. Analysis of the same sample was carried out on three different occasions. The HA values were 45.5 ± 2.0 and 19.1 ± 1.2 μg of uronic acid/mg in the comb and wattle, respectively.

This method was developed to provide a relatively simple, fast assay of HA by introducing shorter time acetone drying and proteolysis of tissues and eliminating the deproteinization procedure. Acetone drying of tissues is commonly carried out for 1 day or longer (Fransson and Havsmark, 1970; Brandt, 1974). However, if the tissue is thin (1 mm in the present study), dehydration appears to be efficient and fast. From our experience, the contents of dry matter of comb and wattle resulting from short (30 min) and long (2 day) treatments, each with three changes of acetone, were similar (Nakano *et al.*, unpublished results). We have also tested the present assay directly in wet tissues of comb and wattle. The HA concentrations determined after proteolysis without deproteinization were 4.5 ± 0.3 and 2.4 ± 0.2 μg of uronic acid/mg of wet comb and wattle tissues, respectively. These values were similar to those for wet tissues calculated on the basis of their proportions of dry matter contents (4.0 ± 0.2 and 2.3 ± 0.2 μg of uronic acid/mg of comb and wattle tissues, respectively). However, the papain digest of wattle tissue without acetone drying tended to have broader electrophoretic bands than did those treated with acetone, providing higher background absorbance. This effect was less prominent in the comb digest. It was concluded that acetone drying, which reduces the broadness of the HA band, is preferable. Acetone removes a considerable amount of lipid, which may be related to the broadness of the HA band.

For papain digestion, tissues were usually incubated overnight for 2 days (Rodén *et al.*, 1972). However, Sweet *et al.* (1976) digested cartilage with papain in 4 h. For the small pieces of comb and wattle tissues, 4 h was found to be sufficient. Deproteinization of papain digests was not necessary for the present analysis. With the conventional method (Rodén *et al.*, 1972; Taniguchi, 1982) involving acetone drying, proteolysis, and deproteinization with trichloroacetic acid requiring subsequent dialysis, it takes a week or more to determine the HA content in the comb and wattle tissues. With the present method, the concentration of HA in these tissues could be determined within a day. This method is also inexpensive, specific, and sensitive. Less than 10 μg of HA can be determined by the present method. Techniques other than cellulose acetate electrophoresis (e.g., ion-exchange chromatography and fractionation with cetylpyridinium chloride–GAG complex) to separate HA require larger amounts of samples and longer time, yet results obtained by these methods are semiquantitative in nature. Jourdian *et al.* (1979) reported a method of HA determination by measuring the amount of oligosaccharides released after digestion with *Streptomyces* hyaluronidase by the periodate–thiobarbituric acid reaction. However, this method requires prior isolation of GAGs free from sialic acid, which is a periodate–thiobarbituric acid reactive material (Warren, 1959). Comb and wattle contain significant amounts of sialic acid (Nakano *et al.*, unpublished results). Thus, the use of *Streptomyces* hyaluronidase digestion may be unpractical for the present purpose.

The method described in this paper will be useful to monitor recovery of HA during isolation of this GAG

from comb and wattle tissues as well as for metabolic and physiological studies of these male-dominated excrescences.

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