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Short communication

A simple method for hyaluronic acid quantification in culture broth

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ABSTRACT

The carbazole assay has been used for determination of the percentage of hyaluronic acid in biological fluids. However, it is difficult to measure the concentration of hyaluronic acid in culture broth because glucose and polysaccharides remaining after cultures can react with sulfuric acid and carbazole. The glucose and polysaccharide remnants must be completely removed in order to get the correct value for hyaluronic acid. The turbidity assay, another method for estimating the concentration of hyaluronic acid, is based on the formation of insoluble complexes between hyaluronic acid and cetyltrimethylammonium bromide. This method is very easy and fast compared with the carbazole assay. Because concentrations of hyaluronic acid measured by the turbidity assay were ranged around 100% of those measured by the carbazole assay, the content of hyaluronic acid in culture broth can be determined by the turbidity assay. The turbidity method also has the advantage of being safer than the carbazole assay.

A number of carbohydrates can be detected by means of a reaction with carbazole. And the reaction of uronic acid with carbazole is the most satisfactory method of measuring uronic acid (Cesaretti, Luppi, Maccari, & Volpi, 2003). It is based on the principle that when carbohydrates are treated with H_2SO_4 or HCl, they yield mixtures of products that react with carbazole to give colors (Bitter & Muir, 1962).

Hyaluronic acid is extracted from rooster combs or produced by microbial fermentation. Because government regulations against the therapeutic usage of animal derived agents are becoming increasingly stringent, it is becoming more attractive to produce hyaluronic acid by microbial fermentation (Yamada & Kawasaki, 2005). There are many materials that react with carbazole in microbial culture broth. These are hyaluronic acid, glucose, and other polysaccharides. The estimation of hyaluronic acid concentration in culture broth must be carried out after performing purification steps to remove material residues.

In general, cells are removed to obtain supernatant and hyaluronic acid is precipitated by the addition of organic solvent. After washing the precipitate with 70% ethanol, the precipitated hyaluronic acid is re-dissolved in distilled water or a buffer. Because it takes a long time to completely dissolve the precipitated hyaluronic acid, the solution of hyaluronic acid must be dissolved in a shaking incubator at 37 °C, overnight. These steps are very laborious and time consuming.

Many polyanions are precipitated by organic ammonium cations, e.g., cetylpyridinium and cetyltrimethylammonium bromide (CTAB). The formation of insoluble complexes between hyaluronic acid and CTAB was described by Scott (1955). In 1956, the hyaluronic acid quantification method was reported by Ferrante (1956). In brief, the insoluble complexes are formed in the mixture of hyaluronic acid and CTAB. The amount of turbidity is proportional to the amount of hyaluronic acid in the system. This method takes 30 min until results are attained. Because the turbidity assay is simpler and less laborious than the carbazole assay, it is better to use the turbidity assay to estimate hyaluronic acid concentration.

The fermentation was carried out in a 7-L jar fermenter with 5-L working volume. The cultivation steps used in this study was followed by the culture method of Kim (Kim, Park, & Kim, 2006), using *Streptococcus zooepidemicus*. The main fermentation medium contained 80 g/l glucose, 5 g/l yeast extract, 17 g/l casein peptone, 0.6 g/l glutamic acid, 0.7 g/l $MgSO_4$, 2.5 g/l K_2HPO_4, 5 g/l NaCl, and 0.5 g/l antifoaming agent. The fermentation broth was diluted 5-fold with distilled water. One volume of 0.1% sodium-dodecyl-sulfate was added to the diluted broth. The mixture was incubated at room temperature for 10 min to free capsular hyaluronic acid. Subsequently, the mixture was filtered (0.2 μm) and used in the turbidity assay (Ferrante, 1956).

However, we modified the amount of samples and reagents. Fifty microliters of hyaluronic acid standard (Sigma, originate from *Streptococcus zooepidemicus*, molecular weight 1,000,000 Da, 1–0.1 mg/ml) of the mixture was placed in a 96-well plate. Fifty microliters of 0.2 M acetate buffer (0.2 M sodium acetate–acetic acid, 0.15 M sodium chloride, pH 6) was added. The plate was incubated at 37 °C in order to synchronize the reaction temperature. And 100 μl of CTAB in 2% sodium hydroxide (incubated at 37 °C) was added carefully. After shaking the plate with a vortex, the plate was read in a plate reader at a wavelength of 600 nm within 10 min because the insoluble complexes can disappear after

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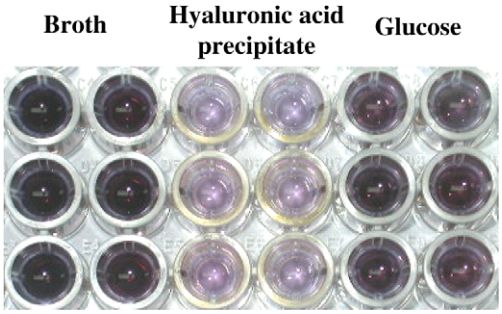

Fig. 1. The 96-well assay for carbazole reaction of the hyaluronic acid samples and the glucose solution.

60 min. These modifications describe an efficient 96-well assay allowing rapid processing of a number of samples. There was no difference of the results when compared with the method of Ferrante. And the residual protein did not affect the turbidity assay because most of the proteins exist as a short chain peptide form derived from the casein peptone (data not shown).

In order to measure hyaluronic acid concentration with carbazole assay, the fermentation broth was diluted 2- or 4-fold with distilled water. And the diluted mixture was filtered (0.2 μm) to remove cells. Subsequently, hyaluronic acid was precipitated from the supernatant by addition of one volume of organic acid, ethanol, or isopropanol. The precipitated hyaluronic acid was washed three times with 70% organic solvent to remove glucose and other soluble sugars that can react with carbazole to give colors. After the washing steps, the precipitate was re-dissolved in an equal volume of 0.2 M acetate buffer and the solution was incubated in a shaking incubator at 37 °C to fully dissolve the precipitate. The purified hyaluronic acid was used for the carbazole assay and the experimental steps described in the following paper (Cesaretti et al., 2003).

Fig. 1 shows a 96-well plate in which the assay of carbazole reaction has been performed. When the broth reacted with carbazole without performing the purification steps, the reaction color was dark black because of other sugars, especially glucose remnants, in the culture broth (Fig. 1). And the 10 mg/ml glucose solution, a concentration of average glucose remnants in the culture broth, showed with black when it reacted with carbazole. But the color of the precipitated hyaluronic acid with the glucose remnants removed was a bright pink. Because the influence of the glucose remnants was excluded by the purification steps, the color of the precipitated hyaluronic acid was brighter than that of the broth and the glucose solution (Fig. 1). Therefore, the precise concentration of hyaluronic acid cannot be measured by the carbazole assay because of the interference of glucose and other sugars in the broth.

Table 1
The content of hyaluronic acid (HA) and glucose in HA samples.

	Broth	Ethanol precipitation[a]	Isopropanol precipitation
HA, carbazole[b], mg/ml	3.49 ± 0.02	2.30 ± 0.05	2.34 ± 0.02
HA, turbidity[c], mg/ml	2.35 ± 0.04	2.34 ± 0.01	2.36 ± 0.02
Glucose, mg/ml	10<	0.001>	0.001>
Carbazole/turbidity × 100, %	149	98	100

[a] Purified by solvent precipitation described in the paper.
[b] Measured by the carbazole assay.
[c] Measured by the turbidity assay.

When the concentration of hyaluronic acid in the broth was estimated by the two methods, the value measured by the carbazole assay was about 50% greater than that measured by the turbidity assay (Table 1). This is due to the glucose remnants, 12.1 mg/ml, that remain in the culture broth after cells used them to metabolize. Because the remnants can react with carbazole, the reaction color was darker than it is when only hyaluronic acid reacts with carbazole. However, when we estimated the concentration of hyaluronic acid after the purification steps, the concentrations of hyaluronic acid that were measured by the carbazole assay and the turbidity assay were same value. And the content of hyaluronic acid of broth and precipitation measured by turbidity assay were also the same. This is because the glucose remnants were removed by the precipitation and washing steps. There was no difference in the organic solvents used for precipitation (Table 1).

In summary, the carbazole assay is the method traditionally used to estimate the concentration of hyaluronic acid in culture broth. But this method is extremely laborious as well as time consuming. Furthermore, it is of some risk because sulfuric acid is used in the process. We propose to use turbidity assay for measuring hyaluronic acid concentration instead of the carbazole assay because the turbidity assay has the advantages of a simple process, takes only about 30 min, and is relatively safe.

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