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Purification of recombinant hyaluronan lyase of *Streptococcus pyogenes* bacteriophage H4489A expressed in *Escherichia coli* and its application for the specific determination of hyaluronan concentration

Pei-Fen Yang, Cheng-Kang Lee *

Department of Chemical Engineering, National Taiwan University of Science and Technology, 43 Keelung Road Sec. 4, Taipei 106, Taiwan, ROC

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Abstract

Hyaluronan (HA) lyase of *Streptococcus pyogenes* bacteriophage was expressed in *Escherichia coli* and purified to homogeneity by immobilized metal affinity chromatography (IMAC). Unlike most bacterial HA lyases, the phage enzyme specifically cleaved HA to unsaturated oligosaccharides which has an optimum absorption at 232 nm. The absorbance of the digestion product reached a limiting value as reaction time increased. The limiting absorbance showed linearity in the range of concentrations 0.05–0.5 mg/mL. Based on this fact, a specific, simple, easy to apply, low cost, and fast enough method was developed for routine determination of HA concentration of a microbial HA production process. This phage HA lyase-based limiting absorbance method has same accuracy and sensitivity as conventional carbazole method for the determination of HA produced from *Streptococcus zooepidemicus* cultivation.

Keywords: Hyaluronan; Phage hyaluronan lyase; Hyaluronan determination; Limiting absorbance; Carbazole method

1. Introduction

Hyaluronan (HA) is a naturally occurring high molecular weight, nonsulfated glycosaminoglycan that has important structural and biological roles in animal tissue. It is composed of 2000–25,000 repeating disaccharide subunits of β-(1-4)-D-glucuronic acid and β-(1-3)-*N*-acetyl-D-glucosamine. HA has principle uses in biomedical field (Swann & Kuo, 1991), such as ophthalimic and orthopaedic surgery. It is also used in eye drops, cosmetics and food products. The volume of this market is 10–20 times greater than medical HA market (Chong, Blank, Mclaughlin, & Nielsen, 2005).

Commercially, HA is produced through extraction from rooster combs or via microbial fermentation. Carbazole method (Bitter & Muir, 1962) is the most often used methods for estimating HA concentration during the extraction and purification processes from various organs, tissues, and fermentation broth because the size of HA present in samples will not affect the results. This colorimetric method is performed in tubes or microplate (Cesaretti, Luppi, Maccari, & Volpi, 2003) with extensive use of reagents and materials. Not only strong acid and high temperature (100 °C) have to be employed, the procedure of this method is also quite tedious. In addition to carbazole method, more sensitive HA disaccharides quantification methods such as high-performance liquid chromatography (HPLC) (Kakehi, Ueda, & Suzuki, 1993; Toyoda, Motoki, & Tanikawa, 1991), capillary electrophoresis (CE) (Karamanos & Hjerpe, 1998; Lamari & Karamanos, 1999), flurophore-assisted carbohydrate electrophoresis (FACE) (Calabro, Hascall, & Midura, 2000; Mahoney, Aplin, Calabro, Hascall, & Day, 2001) have been developed to determine HA concentration. Besides, enzyme-linked immunosorbent assay (ELISA) (Courel et al., 2002; Fosang, Hey, Carney, & Hardingham, 1990; Grigoreas, Anagnostides, & Vynios, 2003; Maeda, Fujita, Sakura, Sakura, & Goto, 1999; Underhill & Zhang, 2000) and radioimmuno assay (RIA) (Laurent & Tengblad, 1980) employing specific HA binding proteins to determine HA concentration have also been developed. These proposed assay methods appears to have some disadvantage in the application to routine analysis. The HA disaccharides quantification methods (HPLC, CE, and FACE) require multienzyme treatment to obtain HA disaccharides and specialized equipment. ELISA or RIA results are affected by the size of HA present in samples or

* Corresponding author. Fax: +886 2 27376644.
E-mail address: cklee@ch.ntust.edu.tw (C.-K. Lee).

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the inability of HA due to its polyvalent negative charge to be immobilized onto solid surface.

Hyaluronan (HA) lyases are produced by many different genera of bacteria (Baker, Yu, Morrison, Averett, & Pritchard, 1997; Hamai, Morikawa, Horie, & Tokuyama, 1989; Hiyama & Okada, 1975; Michelacci & Dietrich, 1976; Ohya & Kaneko, 1970; Shain, Homer, & Beighton, 1996; Yamagata, Saito, Habuchi, & Suzuki, 1968). All Gram-positive bacteria which produce HA lyase to degrade HA and other glycosaminoglycans appear to be capable of causing infections in animals. Possession of HA-degrading activities may facilitate bacterial adhesion, colonization, and invasion of the tissues of an animal host (Kostyukova, Volkova, Ivanova, & Kvetnaya, 1995). Bacterial HA lyase degrades HA by cleaving *N*-acetylglucosamidic bonds of HA by a β-elimination process. The products are unsaturated oligosaccharides, often disaccharides, with a Δ-4,5-uronate residue at nonreducing termini which has an optimum absorption at 232 nm (Yamagata et al., 1968). In addition to HA, some HA lyases also have an ability to cleave other glycosaminoglycan such as chondroitin sulphates in a similar manner (Baker et al., 1997). Several bacteriophages from HA-encapsulated group A streptococci are also known to produce HA lyase (Hynes & Ferretti, 1989; Niemann, Birch-Andersen, Kjems, Mansa, & Stirm, 1976). Recently, HA lyase of *Streptococcus pyogenes* bacteriophage H4489A, unlike most bacterial HA lyases, was found to be exclusively specific for HA (Baker, Dong, & Pritchard, 2002; Hynes & Ferretti, 1989). The only other HA lyase known to be specific for HA is that from *Streptomyces hyalurolyticus*. However, this streptomyce enzyme cleaves HA at a 30-fold slower rate than the phage HA lyase (Baker et al., 2002). Because of its HA-specificity and fast reaction rate, the phage HA lyase is very suitable to be employed for HA determination by digesting HA into unsaturated oligosaccharides. The progress of HA digestion can be monitored by UV absorbance at 232 nm. The obtained limiting absorbance is proportional to the HA concentration. Thus, the phage enzyme-based limiting absorbance method involves no hazardous chemicals and tedious laboring may become a useful alternative choice for HA concentration determination.

In this work, recombinant HA lyase of *S. pyogenes* bacteriophage H4489A was produced from *E. coli*. After purification by immobilized metal affinity chromatography (IMAC), phage HA lyase was employed to develop the limiting absorbance method which is simple, easy to apply, low cost, and fast enough to be used for routine determination of HA content of microbial HA fermentation samples. The results obtained by this phage HA lyase-based limiting absorbance method are not affected by the size of HA present in the samples.

2. Experimental

2.1. Materials

Isopropyl-β-D-thiogalactopyranoside (IPTG) and kanamycin were purchased from Sigma, and sodium hyaluronate was obtained form Fine Chemical Division of Q.P. Co. (Tokyo, Japan). Chondroitin sulfate (CS) sodium salt was obtained from Sigma Co. (St Louis, MO). Protein molecular weight standards were purchased from Gene Mark Technology. DNA marker was purchased from Yeastern Biotech Co. (Taipei, Taiwan). Chelating Sepharose fast flow was obtained from Pharmacia Biotech. All other chemicals were purchased from either Acros or Sigma. Plasmid pSF49, containing the HA lyase *hylP* gene of *S. pyogenes* bacteriophage H4489A, was kindly supplied by Dr Wayne L. Hynes (Department of Biological Science, Old Dominion University, Norfolk, VA).

2.1.1. Phage HA lyase vector construct

The complete phage *hylP* gene was amplified from plasmid pSF49 using a forward primer containing an *Nde*I site and a reverse primer containing a *Not*I site by polymerase chain reaction (PCR). The forward primer (5′-CCGCATATGACTGAA AATATACCATTAAGAGTC-3′) and the reverse primer (5′-CCGGCGGCCGCTC AATGATGATGATGATGATGTT TTTTTAGTATGAGTTTTTTTAA-3′) generated a product 1.08 kb in length. The *hylP* gene fragment obtained from PCR was purified by gel extraction. After digestion with *Nde*I and *Not*I using standard procedures, the *hylP* gene fragment was ligated into a pET30b vector. The product (pHY30) was transformed into *E. coli* TOP10 and selected on Luria-Bertani (LB) medium plates containing 30 μg/mL kanamycin. A plasmid miniprep was sequenced to confirm that the desired product had been obtained. The plasmid pHY30 was then transformed into the expression host *E. coli* BL21(DE3) for phage HA lyase production.

2.2. Expression and purification of HA lyase

The recombinant strain *E. coli* BL21(DE3)/pHY30 was grown overnight at 37 °C in 4 mL LB medium containing 30 μg/mL kanamycin. Aliquot (3 mL) of this starter culture was transferred to 30 mL of the same medium in a 500 mL flask and incubated at 30 °C with vigorous shaking. HA lyase expression was induced by adding IPTG to give a final concentration of 0.1 mM when the culture absorbance at 600 nm reached 0.5. Three hours after induction, the cells were harvested by centrifugation and the cells pellet was washed twice with 30 mL pH 7.5, 0.1 M phosphate buffer. The cells pellet was resuspended in the phosphate buffer and lysed using an ultrasonic cell disruptor (Microson model XL200). The sample was in an ice-bath, and sonication was carried out in short bursts in order to avoid overheating the mixture. The lysate was centrifuged at 16,000*g* for 10 min at 4 °C. Since the expressed HA lyase was fused with a 6×His tag at its C-terminal, the supernatant of the cell lysate was loaded onto a fast-flow chelating Sepharose (Amersham Pharmacia, Sweden) column for immobilized metal affinity chromatography (IMAC) purification following the protocol provided by the manufacturer.

2.3. Analytical methods

SDS-PAGE analysis was carried out under reducing conditions using a 12% polyacrylamide gel. A Mini protein II gel system (Bio-Rad Laboratories, Richmond, CA) was used to run the gels, which were then stained with Coomassie blue. The HA lyase activity defined by the ability of breaking down HA was measured using spectrophotometry method described by Pritchard, Lin, Willingham, and Baker (1994) with some modifications. Briefly, a 10 μL enzyme solution (0.22 mg/mL) was added to a cuvette equilibrated at 25 °C containing HA (0.4 mg) in 0.4 mL of pH 6.8, 50 mM ammonium acetate buffer. The rate of release of unsaturated oligosaccharides by the enzyme from HA, measured as the rate of increase in absorbance at 232 nm, was measured by using a Jasco V-530 spectrophotometer. The amount of the HA lyase that releases 1 μmol of the unsaturated oligosaccharides product at 25 °C was considered as one enzyme unit. An adsorption coefficient of the digestion product of 5.5×10^3 mM^{-1} cm^{-1} was used (Yamagata et al., 1968). For the study of pH effect on HA lyase activity, ammonium acetate buffer (pH 5–7) and glycine buffer (pH 8 and 9) were employed to prepare HA solutions of different pH. The buffer concentration was kept at 50 mM. The protein concentration was determined by Bradford protein assay with bovine serum albumin as standard.

2.4. HA concentration determination

For the carbazole method, a serial dilution of standard or sample of 150 μL was added into 900 μL of a solution of 25 mM sodium tetraborate in sulfuric acid. The mixture in the test tube was heated for 10 min at 100 °C in an aluminous heating block. After cooling in an ice bath for 15 min, 30 μL of 0.125% carbazole in absolute ethanol were carefully added. After heating at 100 °C for 10 min and cooling in an ice bath for 15 min, the absorbance of the reaction solution was measured at a wavelength of 525 nm. For the determination of HA concentration using HA lyase, 10 μL purified HA lyase solution (0.22 mg/mL) was added into a serial dilution of HA standard or sample solution of 900 μL in pH 6.8, 50 mM ammonium acetate buffer and the progress of HA digestion at room temperature was monitored at 232 nm. The limiting absorbance was used for establishing calibration curve. The limiting absorbance was defined as absorbance at which the absorbance increasing rate is less than 0.003 OD/min. The digestion of chondroitin 6-sulfate with phage HA lyase was also carried out by following the same procedure.

2.5. HA production and sample preparation

Streptococcus zooepidemicus (BCRC 910017) purchased from Bioresource Collection and Research Center (Hsin Chu, Taiwan) was grown at 37 °C in Todd-Hewitt broth for 16 h. For the isolation of HA from *S. zooepidemicus* culture, an equal volume of 0.1% (w/v) sodium dodecyl sulfate (SDS) solution was first mixed with the culture sample for 10 min to dissolve the capsular HA from cells surface (Chong & Nielsen, 2003). After centrifuging at 16,000*g* for 10 min to remove the cells, 3-fold volume of 95% ethanol was mixed with the supernatant to precipitate HA. The alcohol precipitated HA was dissolved in the assay buffers for concentration determination by either carbazole or phage HA lyase-based limiting absorbance method.

3. Results and discussion

3.1. Expression and purification of recombinant phage hyaluronan lyase

SDS-PAGE was employed to analyze the expression of phage HA lyase in the *E. coli* BL21(DE3) harboring plasmid pHY30. As shown in Fig. 1, under 0.1 mM IPTG induction a predominant protein band at around 40 kDa was observed in both soluble and insoluble fractions. More than 50% of the over-expressed protein existed as insoluble form. The mass of this induced protein closely corresponded to the expected molecular mass of 40,338 Da which included the 6×His tag region. Compared with the molecular mass of bacterial HA lyases (77–121 kDa) (Hynes & Walton, 2000) that of the phage HA lyase was notably small. Since a 6×His tag was designed to be at the C-terminal of the phage HA lyase in the construct pHY30, the purification of 6×His tagged phage HA lyase from crude cell extract was easily achieved by employing metal chelating affinity chromatography. It was purified to at least 90% purity based on SDS-PAGE analysis. The high concentration of imidazole (0.5 M) employed for eluting the bounded HA lyase was found not to affect its activity at all (data not shown). As shown in the purification table (Table 1), a single metal chelating affinity purification step resulted in a 57-fold increase in purity and an activity recovery yield of 130%. The higher than 100% activity recovery yield represents that the activity of the HA lyase in the crude extract is lower than that found in the column eluate. We found that the activity of purified HA lyase will be reduced by mixing with the crude extract of host *E. coli* cells. Probably, the high molecular weight impurities in the crude extract may complex with

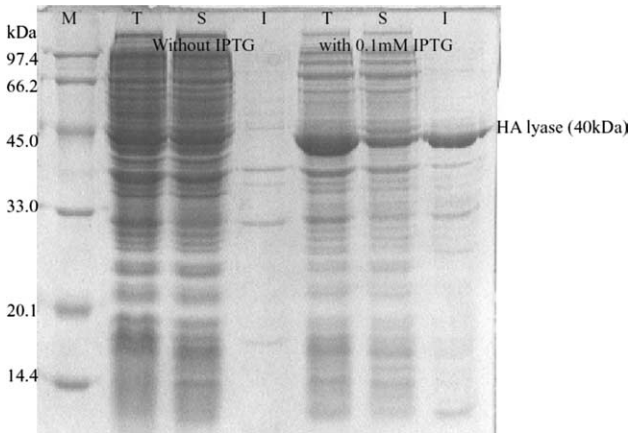

Fig. 1. SDS-polyacrylamide gel electrophoresis analysis of recombinant phage HA lyase expressed by *E. coli* BL21(DE3) harboring pHY30 with/without IPTG induction. T, total cell protein; S, soluble fraction; I, insoluble fraction.

Table 1
Purification of recombinant phage hyaluronate lyase by single-step chromatography on a metal-chelating affinity column from a 30 mL *E. coli* culture

	Protein (mg)	Specific activity (U/mg)	Total activity (U)	Yield (%)	Purification (fold)
Crude extract	9.41	0.17	1.56	100	1
Column eluate	0.22	9.46	2.10	134	57.1

the polyvalent negative charge HA that hinders the reaction between HA and HA lyase and results in a lower HA cleavage rate. It may also possible that the higher than 100% activity recovery yield was due to the presence of HA lyase inhibitors in the crude extracts that were removed after purification. Compared with the specific activity of HA lyase of *S. pneumoniae* expressed in *E. coli* and purified by the same IMAC method (Jedrzejas, Mewbourne, Chantalat, & McPherson, 1998), the specific activity of purified phage HA lyase is about 500-fold lower. The same trend of result has been reported by Baker et al. (2002) that the turnover number of this phage HA lyase is 1700-fold lower that of HA lyase from group B streptococci. The relatively low rate of HA cleavage by the phage enzyme suggests that its mode of action on cleaving HA is very different from bacterial HA lyases. It cleaves HA with a distributive rather than processive action pathway (Baker et al., 2002). Fig. 2 shows the pH effect on phage HA lyase activity. At room temperature the phage enzyme was most active at pH 6.0 that is similar to the optimum pH of *Streptococcus pneumoniae* HA lyase (Jedrzejas et al., 1998). Besides, the purified phage HA lyase was quite stable since its activity declined 15% after 5 days incubation at room temperature (data not shown).

3.2. Determination of HA concentration by phage hyaluronan lyase

The typical HA digestion progress curves carried out by phage HA lyase are shown in Fig. 3. The absorbance at 232 nm increased linearly in initial stage and leveled off as the reaction

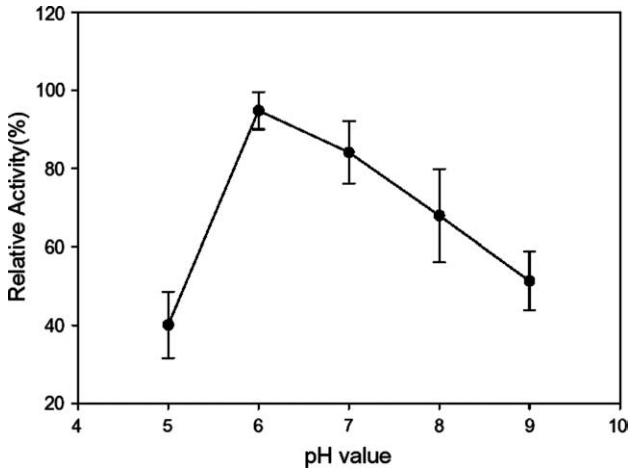

Fig. 2. Effect of pH on phage HA lyase activity at room temperature.

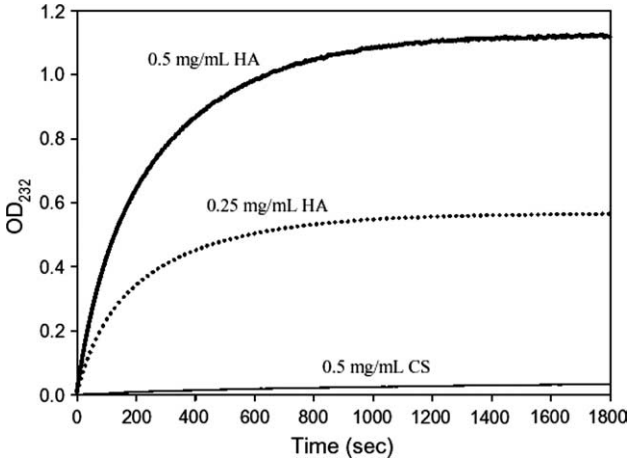

Fig. 3. Typical HA and chondroitin 6-sulfate (CS) digestion progress curves carried out by phage HA lyase. Ten microliter purified phage HA lyase of 0.22 mg/mL was mixed with 900 μL HA or CS solution.

time prolonged. The increase in absorbance at 232 nm indicated the cleavage of HA by elimination, in contrast to hydrolysis, to produce unsaturated uronosyl residues. Unlike most HA lyases, this recombinant phage enzyme does not posses chondroitin lyase activity. As shown in Fig. 3, no appreciable increase in absorbance at 232 nm was noted when chondroitin 6-sulfate (CS), homologue of HA was employed as substrate. When HA concentration of 0.25 mg/mL was digested with 10 μL of purified enzyme (0.22 mg/mL), a limiting absorbance about 0.55 was obtained after 10 min. Once HA concentration was doubled to 0.5 mg/mL, the limiting absorbance was also doubled to about 1.1.

Calibration curves were constructed by using the phage HA lyase-based limiting absorbance method and conventional carbazole method. As shown in Fig. 4, both methods showed nearly the same linear functions (coefficient of correlation >0.99) in the range of HA concentrations 0.05–0.5 mg/mL. A linear calibration curve for chondroitin 6-sulfate could also be established by carbazole method. Due to the specificity of this phage HA lyase, the limiting absorbance

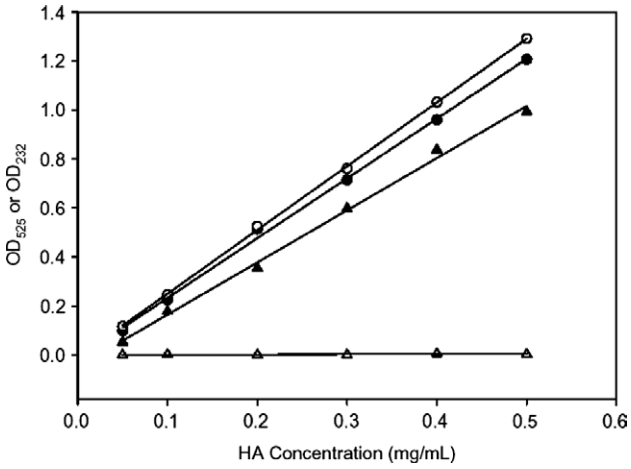

Fig. 4. Calibration curves established by carbazole (OD_{525}; filled symbol) and phage HA lyase-based limiting absorbance (OD_{232}; open symbol) methods for HA (○,●) and CS (△,▲) concentration determination.

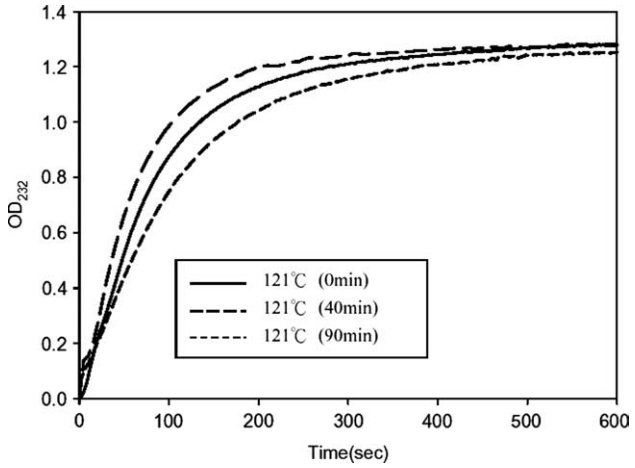

Fig. 5. The HA size effect on HA digestion progress curves. HA samples were prepared by autoclaving 0.5 mg/mL HA solution for different times.

method based on phage enzyme could not be applied to measure CS concentration. Compared with other HA binding protein-based assays (Courel et al., 2002; Fosang et al., 1990; Grigoreas et al., 2003; Maeda et al., 1999; Underhill & Zhang, 2000), the sensitivity of the phage HA lyase-based limiting absorbance method is quite low. It is not suitable for measuring HA level in body fluid (<100 ng/mL). However, its sensitivity is well above that required for measuring HA concentration in the production and purification of HA from microbial fermentation or animal sources. Its result has the same accuracy as that of conventional carbazole method.

Fig. 5 shows the HA lyase digestion progress curves of thermally degraded HA samples. The samples were prepared by autoclaving HA solution (0.5 mg/mL) for 40 and 90 min. It has been reported that the molecular weight of HA will decrease at least 20% after 100 min refluxing 1% (w/v) HA solution (Drimalova, Velebny, Sasinkova, Hromadkova, & Ebringerova, 2005). Heating at higher temperature (121 °C in this work) probably will decrease HA molecular weight further. As we can see from the digestion curves, HA of different degradation extents resulted in very close limiting absorbances with standard deviation about 2%. The insensitivity of HA size effect on limiting absorbance is resulted from the fact that HA of different sizes but with the same weight concentration will be digested to the same extent (e.g. disaccharide) and produces the same amount of unsaturated residues as reaction time prolonged.

In order to test its practical application, this limiting absorbance method was used to determine HA content in the fermentation broth of *S. zooepidemicus* (Fig. 6). HA from fermentation broth was isolated by using ethanol precipitation and assayed simultaneously by limiting absorbance and carbazole methods. As indicated by the calibration curves (Fig. 4), the HA concentrations determined by limiting absorbance method were close to that measured by carbazole method. Thus, the phage HA lyase-based limiting absorbance method provides a practical alternative to specific determination of HA content in microbial HA production processes. Like the carbazole method, this method also requires HA in the culture medium to be isolated before carrying out the assay. We have tried to directly assay HA content in the culture medium without HA isolation. However, the culture medium caused significant interference in the spectrophotometer reading and no reliable data could be obtained. The ethanol precipitation of HA employed for carbazole method was adopted in the phage HA lyase-based limiting absorbance method.

4. Conclusions

The results suggested that the phage HA lyase-based limiting absorbance method is a specific, simple, easy to

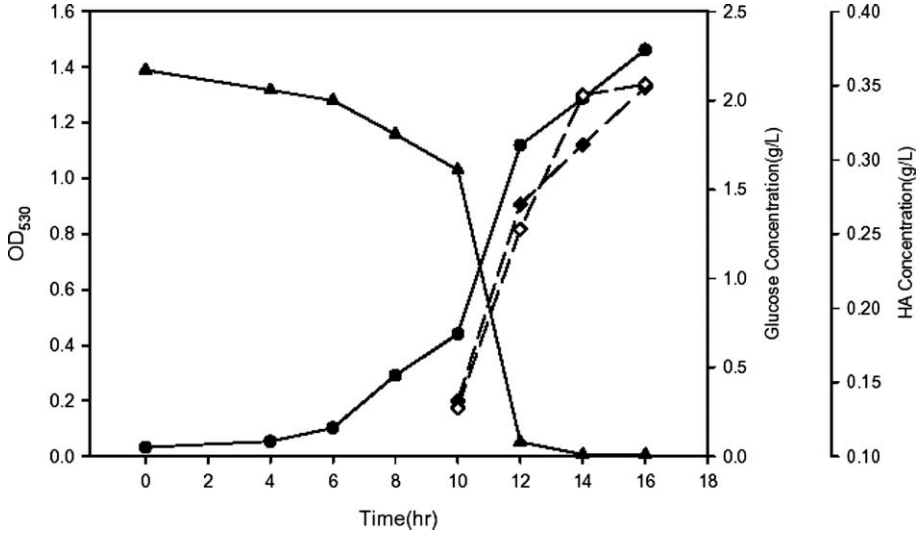

Fig. 6. The growth curve of a *Streptococcus zooepidemicus* culture. (●) cell concentration determined as optical density at 530 nm; (▲) glucose concentration; (♦) HA concentration measured by carbazole method; (◇) HA concentration measured by the limiting absorbance method.

apply, low cost, and fast enough method for routine determination of HA concentration of a microbial HA production process. It has same accuracy and sensitivity as conventional carbazole method for HA concentration determination. Its results are not affected by the size of HA present in the samples. However, like carbazole method, a sample pretreatment procedure is required for this method to isolate HA in order to obtain reliable measurement. Therefore, an efficient sample pretreatment procedure needs to be developed to make the whole HA assay process more effective.

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