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# Characterization of enzymatically digested hyaluronic acid using NMR, Raman, IR, and UV–Vis spectroscopies

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**Abstract**

Hyaluronic acid (HA) is a linear polysaccharide formed from disaccharide units containing *N*-acetylglucosamine and glucuronic acid. When HA was digested with the enzyme hyaluronidase, a double bond is formed. It is known that this double bond forms a complex (radical scavenger) with the radicals (ROO, HO) during UV irradiation, and reduced the toxicity of the radicals before they are absorbed in the human skin. Therefore, the characterization of the double bond formed after the enzymatic digestion of HA is very important. In this study, $^{1}$H NMR, $^{13}$C NMR, Raman, infrared (IR), and UV–Vis spectroscopies were used for characterization of the double bond of HA after enzymatic digestion. HA derivatives in shape of films were tested using Raman and infrared (IR) spectroscopies and the wavenumber of the double bond and some other assignment were determined. The $^{1}$H and $^{13}$C NMR spectra were measured for HA derivatives in $D_2O$ solutions. The chemical shifts and coupling constant of $^{1}$H and $^{13}$C were assigned to the CH=C fragment. The relative amount of olefinic proportion in the mixture was obtained from $^{1}$H and $^{13}$C NMR spectra. The spectroscopy measurement showed an increase in the double bond amount with increasing enzymatic digestion time.


*Keywords:* Hyaluronic acid; NMR spectroscopy; Raman spectroscopy; IR spectroscopy; UV–Vis spectroscopy

## 1. Introduction

Hyaluronic acid (HA, Fig. 1) was isolated for the first time from vitreous humor of eyes by Meyer in 1934 [1]. Its molecular weight is usually in the order of $10^6$–$10^7$ [2]. In aqueous solution, HA has secondary structure and because of the hydrogen bonding which is responsible for the stiffness of its chain, it behaves as expanded random coil with a diameter of 500 nm. The chain entangled already at concentrations in the order of 1 g/l [3]. HA is present in the body, in the skin, vitreous humor, cartilage, and synovial fluid [1,4]. It is used as a diagnostic factor for many diseases such as tumor, rheumatoid arthritis, and liver

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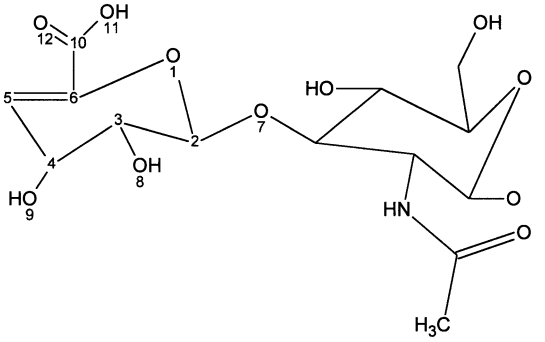


Fig. 1. HA structure after enzymatic digestion.

diseases [5]. In addition, HA is injected to patients with joint diseases and used in ophthalmological and otological surgeries (Visco-surgery) [6]. Foschi et al. [7] described that HA is a radical scavenger. It was also reported that enzymatically digested HA showed more capacity as radical scavenger than intact HA [8]. Moreover, the bioavailability of low molecular weight HA seems to be higher than intact HA [9]. Therefore, the characterization of HA structure after the enzymatic digestion is very important. Many studies were conducted in order to characterize intact HA structure using NMR and IR spectroscopies. Bjarne et al. [10] investigated the $^{1}H$ and $^{13}C$ NMR spectra of the ethyl, benzylester, tetrabutylammonium, and tetraethylammonium salt of HA in methylsulfoxide ($Me_2SO$-$d_6$) using 1D and 2D techniques. Several NMR studies have focussed on the role of the amide group in the secondary structure of oligosaccharides derived from HA [11–14]. The $^{1}H$ and $^{13}C$ NMR spectra of hyaluronate in aqueous solution have been assigned for different pH values and temperature [15–17]. IR spectroscopy and optical microscopy have been performed as a function of relative humidity on wet-spun oriented films of derivatives of HA prepared with various counterions [18]. Barbucci et al. [19] analyzed three HA derivatives with different types of esterification by means of static contact angle measurements, IR spectroscopy, electron spectroscopy, electron microscopy, and thermal analysis. But no Raman spectroscopy data on HA have been published. This study was conducted to characterize HA after enzymatic digestion with respect to its structure using IR, Raman, UV–Vis, and $^{1}H$ and $^{13}C$ NMR spectroscopies.

## 2. Experimental

### 2.1. Materials

HA derivatives [8] with molecular weights of 31, 235, and 1200 kDa were obtained from Hans-Knöll-Institut, Jena, Germany. $D_2O$ and $Me_2SO$-$d_6$ were obtained from Aldrich (USA).

### 2.2. NMR spectroscopy

The $^{1}H$ and $^{13}C$ NMR spectra were measured in $D_2O$ solutions using a VARIAN UNITY 500 spectrometer operating at frequencies of 499.8 and 125.7 MHz, respectively. Chemical shifts were reported as $\delta$ values in ppm relative to 3-trimethylsilylpropionate (TSP), Na salt. Samples were kept in 528-PP Wilmad 5 mm NMR tubes. The NMR spectra were accumulated at 27 °C. The 2D spectra were recorded using the standard VARIAN Software. Correlated spectroscopy (H,H-COSY, GHMQC, GHMBC) spectra were recorded in the absolute value mode and sinebell multiplication was applied in both time dimensions.

### 2.3. FTIR-ATR spectroscopy

The IR spectra were acquired by using a Bruker Spectrometer IFS 28 (Karlsruhe, Germany) equipped with a Spectra-Tech Single-Bounce HART attachment (Shelton, CT). This sampling compartment is a single-reflection ATR accessory that uses a ZnSe crystal with an angle of incidence of 45 ° in horizontal orientation. HA derivatives were measured as films by ATR spectroscopy. These films were produced as follows: because of the difference in the viscosity among aqueous solutions of different molecular weight HA, different concentrations (2% of 1200 kDa HA, 5% of 235 kDa HA, 166% of 31 kDa HA) were prepared and flowed on a glass plates. Then they were placed in an autoclave at 60 °C for 6 h until completely dried.

### 2.4. Raman spectroscopy

The Raman measurements were acquired by using spectrometer RFS 100/S (Brucker, Karlsruhe, Germany). HA derivatives were performed as film as described in Section 2.3.

### 2.5. Diode array spectrophotometer

HP 8452 A (Hewlett-Packard, Waldbronn, Germany) was used to determine UV–Vis spectrum of HA in aqueous solution, which was prepared and degassed using ultrasound bath for 30 min prior to measurement.

## 3. Results and discussion

At different enzymatic digestion time, the molecular weight, structure, and physicochemical characteristics such as solubility and viscosity of HA vary. Characterization of double bonding in the structure of HA with molecular weights of 31, 235, and 1200 kDa after enzymatic digestion was done using $^{1}$H NMR, $^{13}$C NMR, Raman, IR, and UV–Vis spectroscopies. The double bond in pyrancarboxylic acid ring is necessary for reducing the toxicity of radicals during the UV irradiation [20].

### 3.1. NMR spectroscopy

We have investigated the mixture oligomers with $^{1}$H and $^{13}$C NMR spectroscopies in $D_2O$. In $D_2O$ solvent, all exchangeable protons from OH and NH groups were invisible in the $^{1}$H spectrum. The $^{1}$H and $^{13}$C NMR spectra of oligosaccharides have been assigned only partially due to the overlap of several signals from the mixture. The $^{1}$H resonances of the pyrancarboxylic acid ring were assigned according to a COSY 45 experiment (Fig. 2). Carbon shifts of the CH resonances were assigned by H,C shift correlation spectroscopy (GHMQC). It was also possible to identify the structure of the carbons (C-6, C-10) with the obtained coupling pattern of the C,H long-range couplings in the GHMBC spectrum. The observed $^{1}$H chemical shift of $J_{H\text{-}5} = 5.77$ ppm and $^{13}$C chemical shifts of $J_{C\text{-}5} = 107.5$ ppm and $J_{C\text{-}6} =$ 144.8 ppm were assigned to the CH=C fragment. The $^{1}J_{C\text{-}5,H}$ coupling constant of 169 Hz was determined from the $^{13}$C-gated decoupling spectrum. This fact indicated the presence of a double bond in pyrancarboxylic acid ring. The relative amount of olefinic proportion was estimated from the integral intensities of the signals in the $^{1}$H and $^{13}$C spectra (Table 1). The integral intensity of the olefinic proton H-5 or carbon C-5, C-6, compared with the integral intensity from all other protons or carbons of the mixture, was used for the calculation of olefinic proportion [21].

### 3.2. IR spectroscopy

The FTIR-ATR spectra in the spectral range 750–4000 $cm^{-1}$ for HA of 31, 235, and 1200 kDa are presented in Fig. 3. HA of 1200 kDa showed a sharp band at 1654 $cm^{-1}$ due to the C=O carboxyl amide I group. After the enzymatic digestion of HA (Fig. 3), the intensity of the band at 1654 increased with increase in the enzymatic digestion time. We therefore concluded that the ratio of the CH=C double bond to C=O carboxyl amide I increases with increasing enzymatic digestion time of HA. In case of HA of 31 kDa, a band was observed at wavenumber of 1550 and 1750 $cm^{-1}$ for the CH=C double bond [22]. The peak at wavenumber of 1637.3 $cm^{-1}$ for the CH=C double bond was not observed in HA of 1200 and 235 kDa. This has confirmed the results obtained using the $^{1}$H and $^{13}$C NMR spectroscopies. The assignment of IR bands for various HA is given in Table 2.

### 3.3. Raman spectroscopy

Raman spectroscopy method was also used for the characterization of double bond of HA after enzymatic digestion. The Raman spectra of various HA (1200, 235, and 31 kDa) are shown in Fig. 4. As depicted in Fig. 4, the spectrum of HA of 31 kDa exhibited a sharp band at wavenumber of 1661 $cm^{-1}$, and the intensity of the band clearly increases with decreasing molecular weight of HA. Hence, this clearly indicated the formation of double bond in HA 31 kDa structure. At 1200 and 235 kDa, weak bands were observed. The

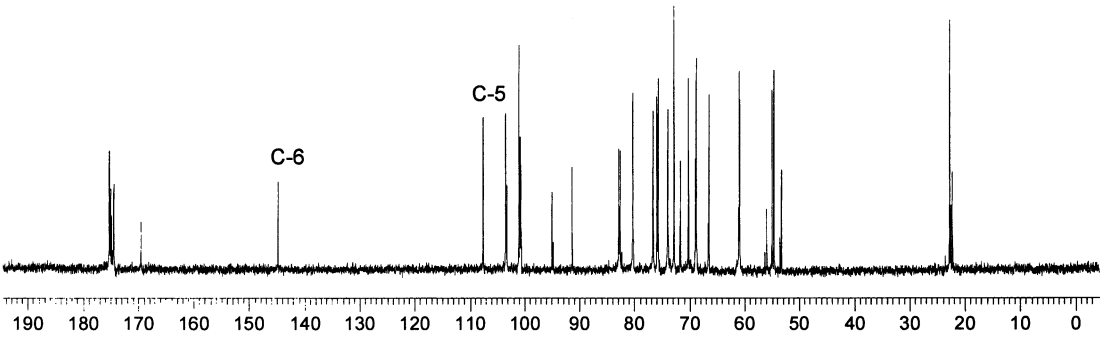


Fig. 2. $^{13}C$ NMR spectrum of 31 kDa HA.

Table 1
The relative amount of olefinic proportion in the mixture

| Compound | Amount of olefinic protons (H-5) by $^{1}H$ NMR (%) | Amount of olefinic C-atoms (C-5, C-6) by $^{13}C$ NMR (%) |
|---|---|---|
| HA (31 kDa) | 2–3 | 5–6 |

weak band at 1630 $cm^{-1}$ corresponds to the amide I group [23]. Table 3 summarizes the Raman bands of HA after enzymatic digestion.

### 3.4. UV–Vis spectroscopy

Further evidence for the presence of double bond in HA after enzymatic hydration was obtained using UV–Vis. HA after enzymatic digestion (31 kDa) showed a maximal absorption at wavelength of 226 nm which demonstrated the presence of the double bond [24]. The absorption at the wavelength of 226 nm was not observed at 235 and 1200 kDa of HA.

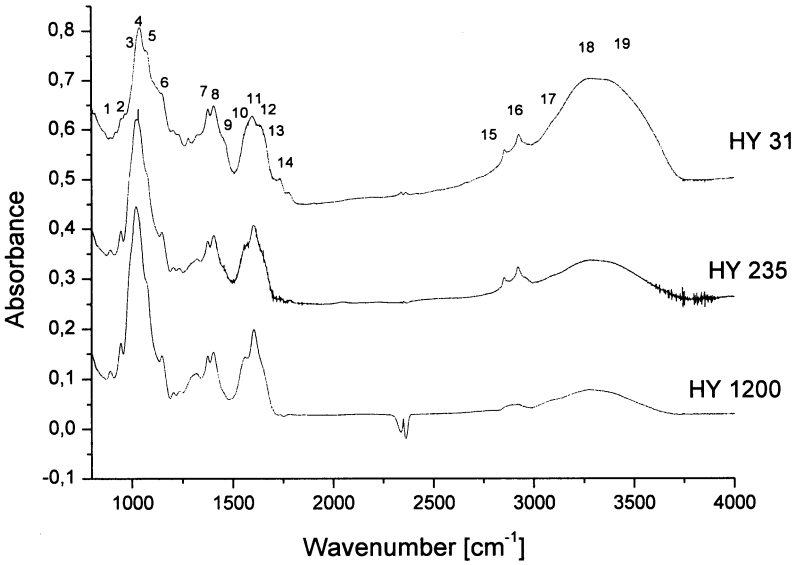


Fig. 3. IR spectra of 31, 235, and 1200 kDa HA.

Table 2
Assignment of IR bands for various HA

| IR bands | | Wavenumber ($cm^{-1}$) | | |
|---|---|---|---|---|
| | | 31 kDa | 235 kDa | 1200 kDa |
| 1 | C–O–C stretching, O–H deformation, and C=O deformation | 896 | 894.5 | 893.9 |
| 2 | | 945.7 | 944.6 | 945.8 |
| 3 | | 999 | 988 | 988 |
| 4 | C–O–C, C–O, and C–O–H stretching | 1038 | 1018 | 1018 |
| 5 | | 1079.5 | 1079 | 1078.6 |
| 6 | | 1155.6 | 1147.9 | 1148 |
| 7 | $CH_2$, $CH_3$, C–O–H deformation, C–O with C=O combination | 1377.7 | 1375 | 1374 |
| 8 | | 1406.9 | 1404.1 | 1404.7 |
| 9 | NH deformation | 1468.7 | 1464 | 1455 |
| 10 | | 1563.7 | 1557.1 | 1559.4 |
| 11 | Amid II | 1597.3 | 1603 | 1602 |
| 12 | | 1637.3 | – | – |
| 13 | C=O carboxyl amid I | 1654.8 | 1655.1 | 1654.1 |
| 14 | | 1736 | – | – |
| 15 | CH stretching | 2853.5 | 2851.8 | 2878 |
| 16 | | 2925 | 2922.4 | 2921 |
| 17 | NH with C=O combination | 3105 | 3105 | 3104 |
| 18 | NH stretching and OH stretching | 3284 | 3284 | 3284 |
| 19 | | 3386 | 3386 | 3386 |

## 4. Conclusion

$^{1}H$ NMR, $^{13}C$ NMR, Raman, IR, and UV–Vis spectroscopies indicated the presence of a double bond in the pyrancarboxylic acid ring after the enzymatic digestion of HA. The double bond in pyrancarboxylic acid ring is necessary for reducing the toxicity of radicals during the UV irradiation

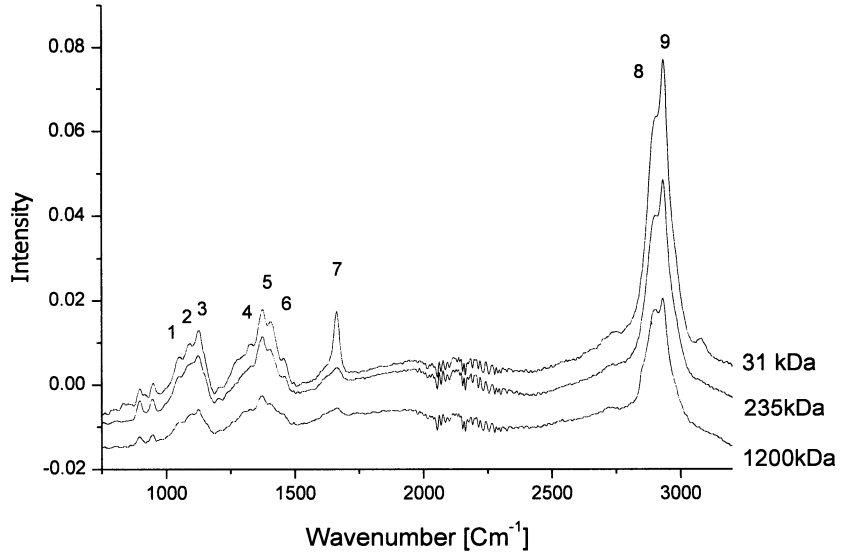


Fig. 4. Raman spectra of 31, 235, and 1200 kDa HA.

Table 3
Assignment of Raman bands for various HA

| Bands | | Wavenumber ($cm^{-1}$) | | |
|---|---|---|---|---|
| | | 31 kDa | 235 kDa | 1200 kDa |
| 1 | C–C and C–O stretching | 1047 | 1047 | 1047 |
| 2 | | 1090 | 1090 | 1090 |
| 3 | | 1122 | 1125 | 1125 |
| 4 | Amid III | 1328 | 1328 | 1328 |
| 5 | C–H bend | 1372 | 1373.5 | 1372 |
| 6 | C–N stretching and C–H deformation | 1405.4 | 1401.4 | 1405.4 |
| 7 | C=C and Amid I | 1662, 1630 | | |
| 8 | CH stretching | 2903 | 2903 | 2903 |
| 9 | N–H stretching | 2934 | 2934 | 2934 |
| 10 | C–H stretching (C from C=C) | 3081 | | |

of the human skin. HA as radical scavenger should be used for the UV protection of the skin. This work gives comprehensive information about the formation of the double bond after the enzymatic digestion of HA.

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