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Synthesis and Characterization of Low Molecular Weight Hyaluronan Sulfates

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Abstract In this study, the sulfate esterification reactions of low molecular weight hyaluronic acid having degree of polymerization (DP) of 48 with SO_3/pyridine (Py) in Py and dimethyl sulfoxide (DMSO) were studied. In the reactions, sulfated hyaluronan samples with degree of substitution (DS) values of 0.22-2.66 were obtainedwith yields of 13.91-78.17%. An increase in the amount of sulfating reagent and the reaction duration in the reaction resulted in an increase in the DS value of the product obtained. In the reactions conducted in DMSO, pronounced polysaccharide chain degradation was observed. The sulfated hyaluronans prepared were structurally characterized by IR- spectroscopic method. Executing the reaction with SO_3/Py / Py system (6.0 mol/mol hyaluronic acid unit (HAU) at 80℃ for 4 h was found to be suitable for the preparation of sulfated hyaluronans having high DS values.

Keywords Hyaluronic acid, Sulfation, Sulfated hyaluronic acid, Structure

1. Introduction

Extraction of sulfated polysaccharides from natural sources requires many costly processes such as extraction, enrichment, complex purification [1]. Also, the fact that natural sulfated polysaccharides have a complex structure prevents the identification of structural-biological interactions in their study [2, pp. 213-259]. A wide range of biological activities is observed not only in natural sulfated polysaccharides, but also in chemically modified sulfate derivatives of polysaccharides [3]. Sulfated polysaccharides can be obtained by the addition of sulfate groups to polysaccharide macromolecules such as cellulose, dextran, pullulan, hyaluronic acid, chitosan, i.e. by chemical sulfation, and they have a number of advantages over their natural analogues [4,5].

2. The Main Findings and Results

Currently, hyaluronic acid with different molecular weight (Mw) values is widely used in many fields, including medicine, cosmetology and food industry [6,7]. Today, the delivery of biologically active compounds to the target organs and the detection of non-toxic, easily absorbed in the body molecules specific to cell receptors are among the main directions in the study of drugs. In this regard, hyaluronic acid is a potential vector in the delivery of anti-tumor drugs due to its toxicity, biodegradability in the body and selective binding to CD44 receptors highly expressed in tumor cells [8,9]. However, due to the high Mw value of natural hyaluronic acid (2-6 mDa), it forms viscous solutions. This, in turn, raises problems with the solubility of drug delivery platforms developed on its basis [10,11]. Small molecular weight hyaluronic acid can be used to solve this problem, but its biodegradation time in the presence of hyaluronidases in the body is very short [12]. This leads to a sharp decrease in the efficiency of delivery of anti-tumor drugs of low molecular weight hyaluronic acid. Recent studies have shown that hyaluronic acid sulfate derivatives inhibit hyaluronidase enzymes [13,14]. This suggests that the study of effective methods for the preparation of low molecular weight sulfated hyaluronic acids is relevant. To date, a number of directions of polysaccharide sulfation reactions have been studied [15]. Polysaccharides can be sulfated under heterogeneous, semi-homogeneous and homogeneous conditions depending on the aggregate state of the reaction medium. H_3SO_4, SO_3, HSO_3Cl, SO_2Cl_2 NH_2SO_3H and SO_3 / amine complexes such as SO_3 / DMF, SO_3 / Py can be used as sulfating reagents [16,17]. In this study, sulfation reactions of a sample of low molecular weight hyaluronic

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acid with SO_3 / Py in a Py (pyridine) and DMSO environment were investigated. In order to determine the optimal reaction conditions, studies were performed with different temperatures, times, and amounts of sulfating reagents. During the reaction, the values of EA (exchange rate) and PD (polymerization rate) of the product, the rate of sulfation, changes in product yield were studied.

3. Materials and Methods

3.1. Sulfation of Hyaluronic acid with Small Molecular Weight

A solution of low molecular weight hyaluronic acid (Mw = 19.3kDa, PD = 48) at a concentration of 25.0 mg / ml in Py or DMSO was prepared. After complete dissolution of the hyaluronic acid in solution, the reaction mixture was placed in an oil bath and SO3 / Py was added at a ratio of 1.0: 1.0-16.0mol / mol hyaluronic acid unit (GKB) to the amount of hyaluronic acid. Reactions were carried out in Py or DMSO solution at 40–80°S for 1–12 h. Upon completion of the reaction, the solution was cooled to room temperature and neutralized using a 0.1 mol / l solution of NaOH (brought to pH 9). To remove the hyaluronic acid sulfate derivatives from the additives, it was dialyzed using a dialysis membrane (MWCO 1000 Da, Spectrum laboratories, USA) and dried using a lyophilic desiccant.

3.2. Determination of Molecular Mass

The values and molecular mass sizes of the samples were determined by gel chromatography. Experiments Jasco PU-980 pump (JASCO GmbH, Germany), Suprema 3000 Å and 30 Å columns (PSS Polymer Standards Service GmbH, Germany), SLD7100 MALLS detector (PSS Polymer Standards Service GmbH, Germany), UV-VIS detector (PSS Polymer) Standards Service GmbH, Germany) and a gel chromatography system equipped with a Jasco RI-930 refractive index (RI) detector (JASCO GmbH, Germany). Determination of MM and MM vi sizes was carried out at 25℃ and an aqueous solution of 0.1 M $NaNO_3$ was used as an eluent. The columns were calibrated to pullulan standards (Sigma-Aldrich Chemie GmbH, Germany).

3.3. Determination of Sulfur Content

The sulfur content of the sulfated samples was determined using EURO EA 3000 (CHNS) (O) Elemental Analyzer (EuroVector Instruments & Software, Italy).

3.4. Study of Structures

The IR spectra of the samples were obtained on a Thermo Nicolet AVATAR 370 FTIR spectrometer, in the absorption range of 400–4000 cm^{-1}, using the KBr tablet method.

4. Results and Their Discussion

Sulfation reactions of low molecular weight hyaluronic acid using SO_3 / Py were performed in Py and DMSO environments. The reaction proceeds by the mechanism of electrophilic exchange between the sulfating reagent and the hyaluronic acid-ON groups. The sulfation reaction of hyaluronic acid with SO_3 /Py is shown in Figure 1.

The studies were performed with small molecular weight hyaluronic acid samples with a PD value of 48 (Mw = 19.3kDa). The studies studied the effect of temperature, reaction duration, amount of sulfating reagent, and environment on the sulfation of low molecular weight hyaluronic acid. Reactions were carried out at a temperature of 40–90℃, for 1–12 h, with an amount of SO3 / Py of 1.0–12.0 mol / mol GKB. During the reaction, the ER and PD values of the product, the rate of sulfation, changes in product yield were studied. The reaction conditions and molecular sizes of the samples obtained are given in Table 1.

It can be seen from the table that the change in temperature under the conditions of sulfation reagent and time unchanged (SO3 / Py 1.0 mol / mol GKB, 1 hour) has a significant effect on the molecular size of the obtained product. The ER values of the product obtained also increased with increasing temperature in the reaction. When the temperature was raised to 80°S, the ER value of the product increased sharply to 0.36, and when the temperature was raised to 90°S, the ER value of the product changed less to 0.37. In this case, an increase in the ER value of sulfated hyaluronic acid with increasing temperature is associated with an increase in the rate of sulfation. This is because the rate of increase of the ER value under the influence of temperature is consistent with the increase in the rate of sulfation. For example, while the rate of sulfation was 0.00605 mol / min at 80°S, this figure was found to be 0.00615 mol / min at 90°S (Figure 2a).

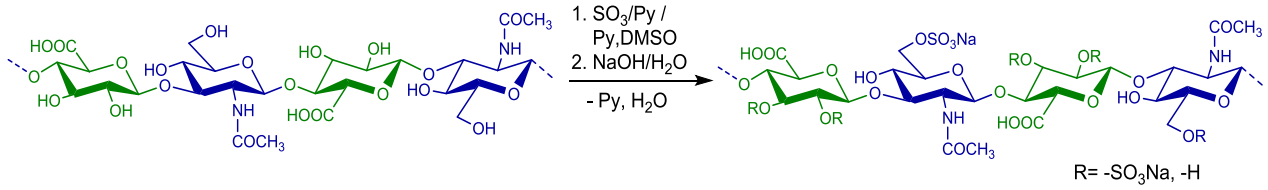

Figure 1. Sulfation reaction of hyaluronic acid with SO_3 / Py in Py or DMSO medium

Table 1. Conditions of sulfation of low molecular weight hyaluronic acid with SO3 / Py in Py and DMSO environment and molecular sizes of the obtained products

Example	Reaction conditions				Results				
	Condition	Py / SO_3 mol / mol GKB	Tem, °C	Time, Hour	PD	S,%	SO_3Na, %	АД	Pro, %
HA	0	0	0	0	48	0,00	0,00	0,00	0
HAS-1	Pyridine	1	40	1	48	1,66	5,34	0,22	13,91
HAS-2	Pyridine	1	50	1	48	1,99	6,41	0,27	17,14
HAS-3	Pyridine	1	60	1	48	2,31	7,44	0,31	20,33
HAS-4	Pyridine	1	70	1	47	2,48	7,98	0,34	23,15
HAS-5	Pyridine	1	80	1	47	2,65	8,53	0,36	25,31
HAS-6	Pyridine	1	90	1	46	2,69	8,66	0,37	25,94
HAS-7	Pyridine	1	80	2	47	3,96	12,75	0,57	31,19
HAS-8	Pyridine	1	80	3	47	4,53	14,58	0,66	35,83
HAS-9	Pyridine	1	80	4	46	4,85	15,61	0,72	37,58
HAS-10	Pyridine	1	80	6	46	5,07	16,32	0,76	39,77
HAS-11	Pyridine	1	80	8	46	5,18	16,67	0,78	40,58
HAS-12	Pyridine	1	80	12	45	5,24	16,87	0,79	41,23
HAS-13	Pyridine	2	80	4	46	7,52	24,21	1,24	51,89
HAS-14	Pyridine	3	80	4	46	9,66	31,09	1,76	63,21
HAS-15	Pyridine	6	80	4	46	11,72	37,72	2,36	73,14
HAS-16	Pyridine	9	80	4	45	12,24	39,40	2,53	75,92
HAS-17	Pyridine	12	80	4	44	12,43	40,01	2,60	77,45
HAS-18	Pyridine	16	80	4	44	12,61	40,59	2,66	78,17
HAS-19	DMSO	1	80	4	43	3,14	10,11	0,44	23,15
HAS-20	DMSO	3	80	4	39	6,54	21,05	1,04	43,24
HAS-21	DMSO	6	80	4	32	8,56	27,55	1,48	55,21
HAS-22	DMSO	9	80	4	25	9,47	30,48	1,71	54,68
HAS-23	DMSO	12	80	4	17	9,86	31,74	1,81	46,47
HAS-24	DMSO	16	80	4	12	10,15	32,67	1,89	35,12

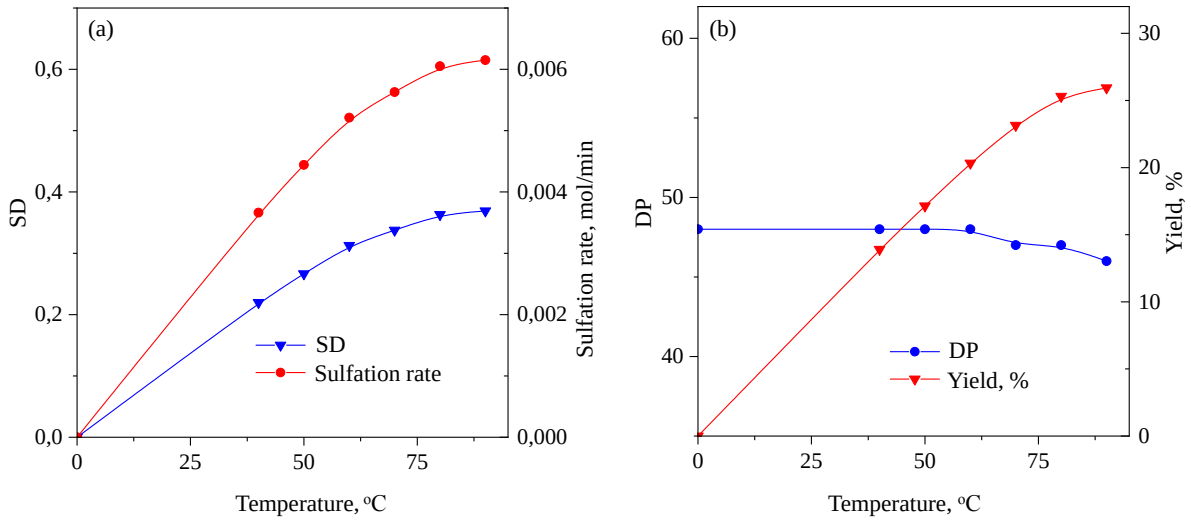

Figure 2. When sulfating hyaluronic acid with SO_3 / Py, changes in ER and sulfation rate (a) and PD and yield (b) under the influence of temperature (SO_3 / Py 1.0 mol / mol GKB, time 1 hour)

Also, increasing the reaction temperature from 40°S to 90oS resulted in a decrease in the PD value of the product obtained from 48 to 46, and the reaction yield was 20.33-25.94%. This condition indicates that a low degree of depolymerization occurs in the polysaccharide chain in the reaction. The ER value, sulfation rate, and yield of the

resulting product were directly proportional to the temperature, and the increase in temperature led to an increase in the values listed above. The growth patterns of these magnitudes were mostly high at 80°S and varied little at 90°C. Therefore, 80°S was selected as the optimum temperature for this sulfation reaction (Fig. 2b).

When a small molecular weight hyaluronic acid sample was sulphated for 12 h under conditions where the amount of sulfating reagent and temperature were unchanged (SO_3 / Py 1.0 mol / mol GKB, 80°S), a sharp increase in the ER value of the samples obtained at the beginning of the reaction was observed. For example, during the first 4 hours, the ER value of the product obtained increased sharply to 0.72, with a subsequent increase in reaction time, a small change in this value was observed, and in 12 hours it was found to increase this value to 0.79. The study also examined the effect of reaction duration on sulfation rate, and found that sulfation rate was also high during the first 4 hours of the reaction and had a maximum value (0.00605 mol / min) during the first 1 hour of the reaction. In the later stages of the reaction, a decrease in the rate of sulfation was observed, and this value was observed to be at minimum values in the range of 6–12 h (Fig. 3a). This indicates that the rate of sulfation was initially high when a sulfating reagent was added to a low molecular weight hyaluronic acid solution. This is due to the high rate of sulfation due to the high content of sulfating reagent and hyaluronic acid-OH groups in the reaction medium in the early stages of the reaction. As the amount of sulfating reagent decreases over time, the rate of sulfation also decreases. For example, the results of the analysis of ER values of the obtained products show that 36.31-72.02% of the amount of SO_3 / Py added to the reaction is consumed in the first 1-4 hours of the reaction. A partial decrease in the PD value of the polysaccharide chain was also observed during the reaction. Also, in this reaction, product yield increased sharply during the first 4 hours and was 37.5%, but changed little in subsequent times, and this figure was found to increase to 41.2% in 12 hours (Figure 3b).

The results obtained showed that the ER value of the product, the rate of sulfation, and the growth rates of yield were high during the first 4 hours and varied little in subsequent times. Therefore, 4 hours is sufficient to sulfate hyaluronic acid with a small molecular mass, thereby preventing excessive depolymerization of the polysaccharide chain.

Also, in the next stage of the study, sulfation reactions were carried out with the amount of SO_3 / Py of 1.0-16.0 mol / mol GKB at the optimum temperature and time (80°S, 4 hours) determined. During the reaction, the effect of the sulfating reagent on the product ER and PD values and yield was studied. The ER values of the product increased with increasing amount of sulfating reagent under conditions where temperature and reaction duration did not change (80°S, 4 h). At the same time, when the amount of SO_3 / Py was increased to 6.0 mol / mol GKB, a sharp increase in ER values of the obtained product was observed to 2.36. Subsequent increases in the amount of sulfonating reagent showed that the ER values changed little, and when the SO_3 / Py content was increased to 16.0 mol / mol GKB, sulfate products with an ER value of 2.66 were obtained (Fig. 4a). The results showed that when the amount of SO_3 / Py was increased to 6.0 mol / mol GKB, the main part (58.95%) of the -OH groups in hyaluronic acid was sulfated. Therefore, with the subsequent increase in the amount of sulfating reagent, the rate of increase of the ER value decreased. In this reaction, it was found that it was possible to synthesize sulfate derivatives with an ER value of 1.0 with a GKB content of 1.0 mol / mol of SO_3 / Py. It was also found that sulphate derivatives with an ER value of 1.0–2.7, respectively, with yields of 2.0–16.0 mol / mol GKB (80°S, 4 h) of the sulfating reagent could be obtained (Table 1). In addition, the effect of the amount of sulfating reagent on the reaction on the product PD values was studied. At the same time, the PD value of the samples decreased with increasing amount of sulfating reagent. However, no sharp decrease in PD value was observed (Fig. 4b).

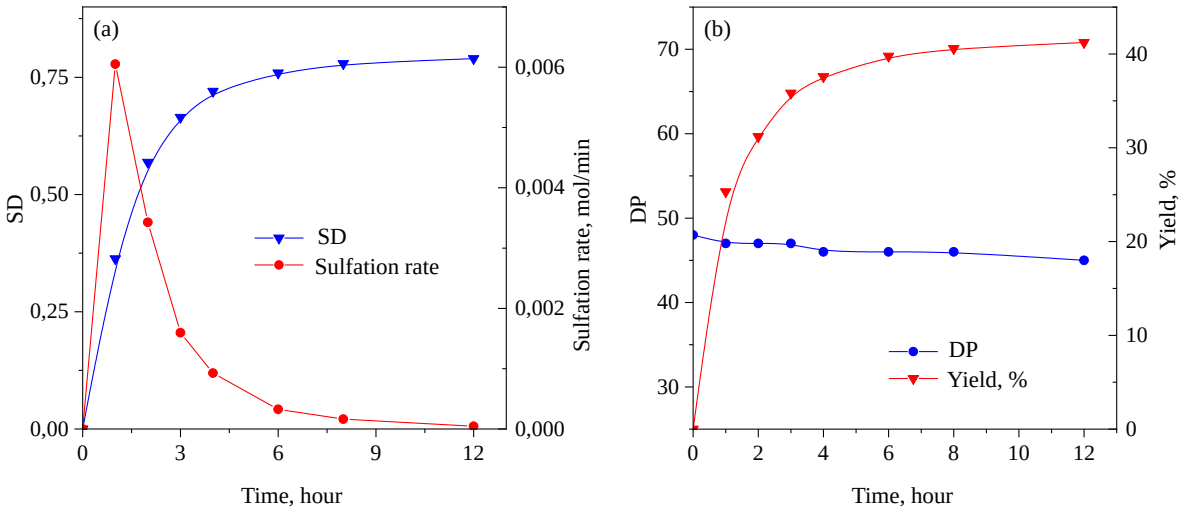

Figure 3. Changes in ER and sulfation rate (a) and PD and yield (b) during sulfation of low molecular weight hyaluronic acid with SO_3 / Py (SO_3 / Py 1.0 mol / mol GKB, temperature 80°S)

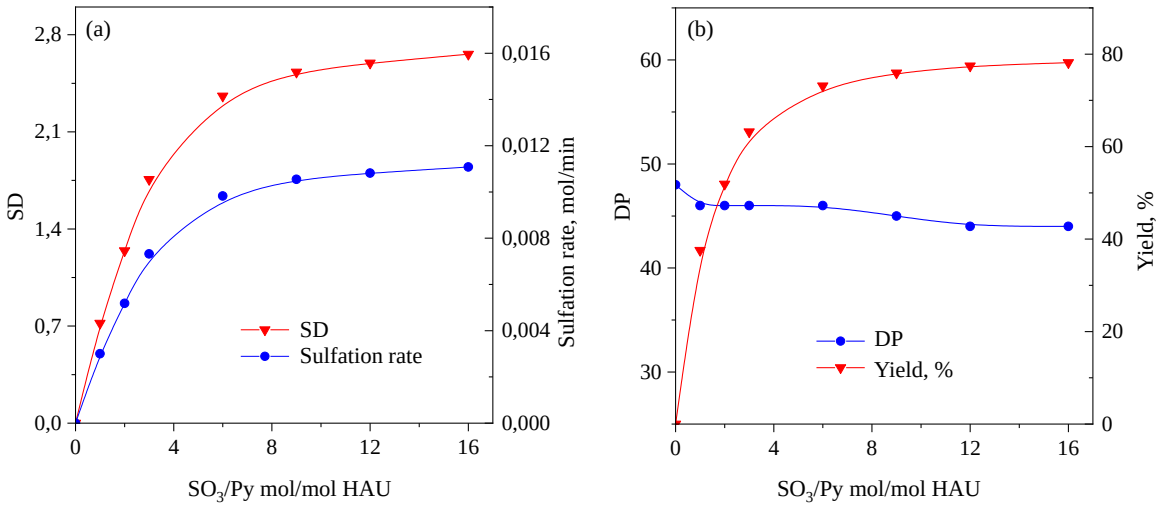

Figure 4. With the increase in the amount of sulfating reagent in the sulfation of small molecular weight hyaluronic acid samples in SO_3 / Py and Py, the change in the rate of sulfation of the product ER (a) and the values of PD and yield (b) (temperature 80°S, time 4 hours)

In the next phase of the study, the effect of the reaction medium on the sulfation reactions was studied. In this case, the reaction was carried out in DMSO medium with different amounts of sulfating reagent. Samples with an ER value of 0.44–1.89 were obtained when the reaction was carried out with an amount of 1.0–16.0 mol / mol GKB of SO_3 / Py under temperature and time-constant conditions (80°S, 4 h) (Fig. 5a). Also, with increasing sulfate reagent SO_3 / Py in the reaction, a sharp increase in the rate of sulfation was observed, followed by a small change. In addition, a sharp decrease in the PD value of the product obtained when low molecular weight hyaluronic acid was sulfated in a DMSO medium was observed (Fig. 5b). This situation can be explained by the sharp depolymerization of the polysaccharide chain under the influence of sulfuric acid, which is formed as a result of partial hydrolysis of SO_3 / Py at the expense of moisture in the reaction medium. In reactions with Py, no deep depolymerization of the polysaccharide chain is observed due to the neutralization of the resulting acid with Py. The results showed that the ER value of the products obtained in the sulfation reactions carried out in the Py medium was higher than that of the samples obtained in the DMSO medium, and the PD values of the products varied less.

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Studies have shown that it is advisable to carry out the sulfation reaction of low molecular weight hyaluronic acid in a Py medium with a sulfating reagent content of 6.0 mol / mol GKB at 80°C for 4 hours.

The structures of hyaluronic acid sulfate derivatives were studied using the IR spectroscopy method. The IR spectra of the first hyaluronic acid and its sulfated product are shown in Figure 6. In the IR spectrum of the samples, the absorption corresponding to the valence oscillations of the O – H and N – H bonds was observed in the 3200-3400 cm^{-1} region of the spectrum. −1 was detected in the field [18]. Absorption in the 1653 cm^{-1} region of the spectrum is characteristic of the valence oscillations of the C = O bonds in the N-acetyl groups, while absorption in the 1611 cm^{-1} region is characteristic of the C = O bonds in the carboxylate ions [19].

It was also found that the absorptions in the 1558 cm^{-1} region of the spectrum corresponded to the C − N (amide I) bonds in the N-acetyl groups. The absorption in the 1409 cm^{-1} region of the spectra corresponds to the deformation oscillations of the C − N (amide II) bonds [20]. Absorption in the 1377 cm^{-1} region of the spectrum is specific for the valence oscillations of C − O bonds in carboxylate ions, while absorption in the 1321 cm^{-1} region is specific for C − N (amide III) bonds in the N-acetyl groups [21]. Absorption in the 1157 cm^{-1} region of the spectrum corresponds to the valence oscillations of the C – O – C bonds in the ring of glucuronic acid and N-acetyl glucosamine residues.

Absorption in the 1003 cm^{-1} region of the spectrum is characteristic of the valence oscillations of C − OH (carbinol) bonds held by hydroxyl groups [22]. Absorption specificity of C – O – C α-b-glycoside bonds between monosaccharide residues in the hyaluronic acid chain was detected in the 856-947 cm^{-1} region of the spectrum. Also, the specific absorption of valence oscillations of C – O – S bonds in sulfated hyaluronic acid was observed at 816 cm^{-1}, and the absorption corresponding to asymmetric valence oscillations of O = S = O bonds was found in the area of 1232 cm^{-1}.

5. Conclusions

Sulfation reactions of low molecular weight hyaluronic acid with SO_3 / Py in Py and DMSO environments were studied. It was found that samples with high ER value (ER = 2.66) can be obtained when sulfation reactions are carried out in SO environment with SO3 / Py. An increase in the amount of sulfating reagent in the reaction led to an increase in the ER value of the product obtained as well as the reaction yield. Studies have shown that it is advisable to carry out the sulfation reaction of low molecular weight hyaluronic acid in a Py medium with a sulfating reagent content of 6.0 mol / mol GKB at 80℃ for 4 hours. The identified optimal conditions can be used in the preparation of sulfate derivatives of hyaluronic acid with a high ER value of small molecular weight.

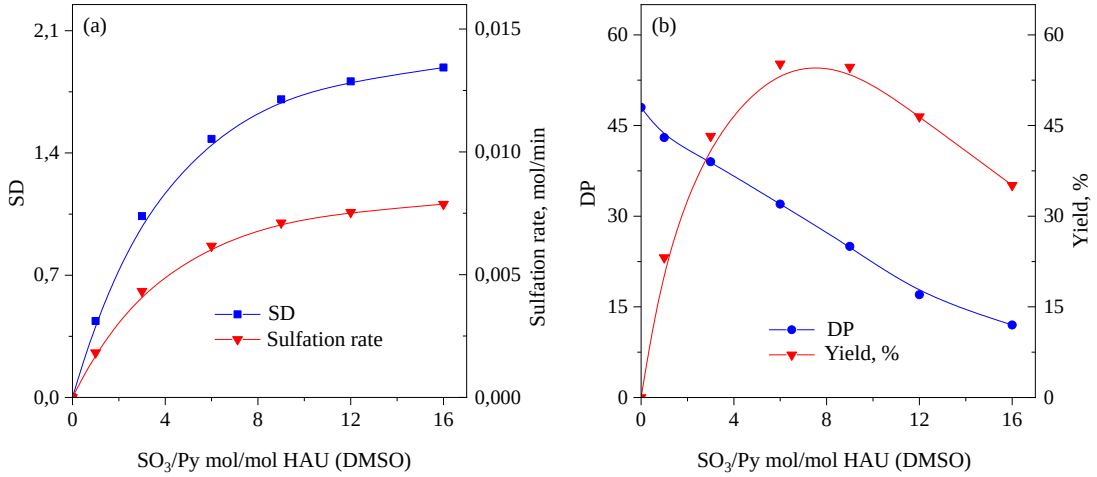

Figure 5. With the increase in the amount of sulfating reagent in the sulfation of small molecular weight hyaluronic acid samples with SO_3 / Py in DMSO, the rate of sulfation of the product ER and sulfide (a) and PD and yield (b) change (temperature 80°S, time 4 hours)

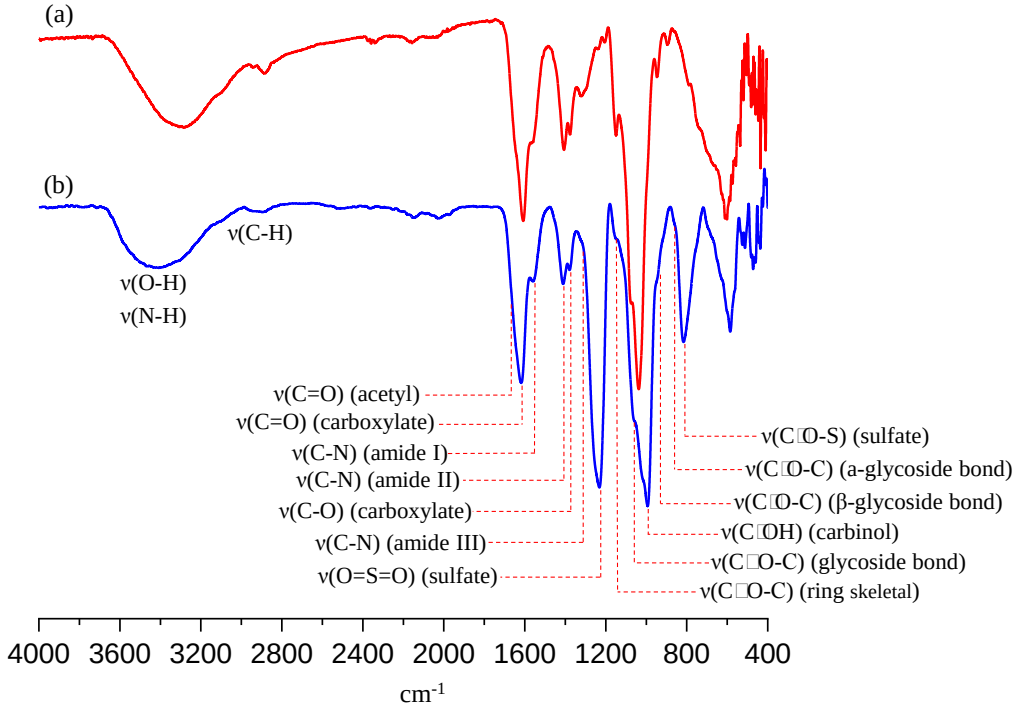

Figure 6. IR spectra of hyaluronic acid and its sulfated product. (In the spectra: (a) hyaluronic acid Mw = 19.3kDa, PD = 48, PDI = 2.46 (b) NAS-15 Mw = 29.5 kDa, PD = 46)

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