Characterization of Poly(β -L-malic acid) Produced by *Aureobasidium* sp. A-91

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ABSTRACT: In a previous paper, black yeast was found to produce poly(L-malic acid) in large yield by fermentation with glucose as the carbon source. Based upon characteristic NMR spectra, the primary structure of this polymer was elucidated as poly(β -L-malic acid) having branched chains. Thermal analysis by DSC shows neither T_g nor T_m below its decomposition temperature T_d on the solid state polymer.

KEY WORDS NMR / DSC / Auto-Catalytic Cleavage / Enzymatic Assay / Oligomer /

Poly(malic acid) is well known as a product by *Penicillium cyclopium*, *Physarum polycephalum*, and *Aureobasidium* sp.¹ In the case of poly(malic acid), there are three different possible isomers:

Poly(malic acid)s of a different structure have been synthesized *in vitro* via direct condensation reaction of malic acid, or by the condensation reaction of α - or β -malic acid benzyl ester to give a specified isomeric polymer followed by debenzylation,^{2,4} but the molecular weight of the polymers thus obtained were limited (less than 4000).

As reported in our previous paper,¹ *Aureobasidium* sp. strain A-91 isolated as black yeast gave rather high molecular weight poly-

(L-malic acid) extracellularly in the course of fermentation with glucose as the carbon source. In the culture broth, pullulan coexisted with this polymer but the poly(malic acid) was easily recovered as its Ca-salt by precipitation with methyl alcohol.

In this paper, we report the elucidation of the primary structure of poly(L-malic acid), based on both the ¹H and ¹³C NMR spectra which were recorded in aqueous solution. NMR spectroscopy was also used to study the auto-catalytic cleavage behavior of the polymer in aqueous solution. Thermal properties of the solid state poly(L-malic acid) were also examined by DSC.

EXPERIMENTAL

Source of Polymalate

In the presence of *Aureobasidium* sp. A-91 isolated in the form of black yeast (the best producer of poly(malic acid)) a culture broth

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was prepared by using a basal medium containing 12% of glucose as the carbon source.¹ During the course of fermentation (one week at 25°C), the pH of the medium was maintained at pH 6.8 by adding CaCO₃.

Isolation and Purification of Poly(malic acid)

The culture broth thus obtained was filtrated through a layer of kieselguhr. After warming up the filtrate (2.3 l, pH 6.4) to 50°C, methyl alcohol (0.8 l) was added dropwise with stirring to precipitate pullulan (*ca.* 2 g) which was removed as a stringy solid by filtration. Methyl alcohol (2.7 l) was further added dropwise and the mixture was left overnight with mild stirring at room temperature. The resulting precipitate (*ca.* 140 g) of Ca-salt of poly(L-malic acid) was collected by decantation.

For the molecular weight fractionation, the Ca-salt of poly(L-malic acid) (80 g) was dissolved into deionized water (1 l) and methyl alcohol (0.5 l) was added dropwise at room temperature to give the highest molecular weight fraction (Fraction 1) (*ca.* 48 g). Further amount of methyl alcohol (0.3 l and 0.7 l) were consecutively added to give Fractions 2 and 3 (25 g and *ca.* 4 g, respectively).

Of these fractions, Fraction 1 had the highest molecular weight (10000) and was used in subsequent experiments for characterization.

To obtain Ca-ion freed poly(L-malic acid), Fraction 1 was dissolved into deionized water and treated with Amberlite IR-120B (H⁺). The aqueous solution thus treated was then lyophilized and dried over P₂O₅ to a constant weight.

Analytical Methods

HPLC was carried out as previously described.¹ ¹H and ¹³C NMR spectra were recorded on D₂O solutions using JEOL-FX 100 and JEOL-GSX 270 spectrometers with DSS or TMS as internal standard references. Differential scanning calorimetry (=DSC) to

observe the thermal properties of the solid state polymer was performed using a SEIKO DSC 220C+SSC 5200 instrument. All DSC samples were carefully sealed in aluminium pans to prevent water absorption. The temperature range scanned was from 25°C to 250°C (with a 10°C min⁻¹ heating rate). The optical rotation of cleavage products were assigned by enzymatic assay using the commercial kits of Boehringer Mannheim GMBH Biochemica, detecting specific activities of L-malate dehydrogenase or D-malate dehydrogenase, respectively.

RESULTS AND DISCUSSION

Elucidation of the Primary Structure of Poly(L-malic acid)

In the case of poly(L-malic acid), there are three possible isomers, α -, β -, and α,β -type as described above. In Table I, previous assignments²⁻⁴ of the NMR spectrum of poly(malic acid) in D₂O are compared with that obtained in this report.

Discrepancies among the values of the chemical shifts from sample to sample might mainly be attributed to the differences in molecular weight and also to the low resolution of the spectra, because they could not detect any signals to be assigned to the terminal structures of the polymers.

It is clear from the literature values of chemical shifts presented in Table I that we are not able to assign each spectrum either to α -type polymer or β -type polymer, consequently the assignment of primary structure was made indirectly. First of all, the NMR spectrum of the D₂O solution of the Ca-salt of poly(L-malic acid) was compared with that of Ca-ion freed poly(L-malic acid) (Figure 1). We expected to observe a significant difference in the chemical shifts brought about by the ability of the Ca-ion freed polymer to behave as an expanded polyelectrolyte chain due to the intramolecular repulsive forces between the charged groups. In contrast, the Ca-salt of

Table I. ^1H and ^{13}C NMR assignments of various poly(malic acid)s in D_2O ^1H NMR

Investigator	Type of poly-(malic acid)	Chemical shifts/ppm		
		Methylene	Methine	Terminal methylene
Otani <i>et al.</i>	α	2.92	5.52	(4.5)
Otani <i>et al.</i>	β	2.99	5.38	
Ouchi <i>et al.</i>	α	2.9	5.5	
Fischer <i>et al.</i>	β	2.9	5.0	
Ours ^a	β	3.1	5.5	4.6

^a (270 MHz) at 24°C. ^{13}C NMR

Investigator	Type of poly-(malic acid)	Chemical shifts/ppm			
		Methylene	Methine	Carbonyl	
				Main chain	Side chain
Otani <i>et al.</i>	α	38.3	72.4	172.6	175.6
Otani <i>et al.</i>	β	38.3	72.4	174.7	176.1
Ouchi <i>et al.</i>	α	38.3	72.5	172.6	175.6
Fischer <i>et al.</i>	β	36.7	72.5	172.3	176.1
Ours ^b	β	38.4, 41.2	71.9, 69.5	173.1	174.6

^b (67.94 MHz) at 24°C.

the polymer behaves as a shranked random coil in aqueous solution. It follows that the chemical shift of the methylene protons of α -type polymer chain might be much affected than those of methylene protons of β -type polymer.

In Figures 1 and 2, it is seen that there is no significant difference in the values of the chemical shifts of the methylene unit in contrast to those of methine unit and of carbonyl unit. Therefore, the fact that a large induced change in chain conformation has little effect on the magnetic environment of the methylene protons, allows the assignment of a β -type structure to be made. Thus the primary structure of this poly(L-malic acid) was deduced as "poly(β -L-malic acid)."

The structure thus elucidated is supported by those data presented especially in Figures 4

(4) and 6 (3) which were assigned to those of monomeric L-malic acid and β -type conjugated L-malic acid in aqueous solutions.

In addition to the structural determination mentioned above, the fine structure of the individual signals of the methine- and methylene-protons were analyzed by NMR spectroscopy on aqueous solutions at different stages of auto-catalytic cleavage.

In Figure 3, comparison of individual signals appearing on the NMR spectra at different stages of cleavage enabled us to assign signals with fine structures to the terminal methine-, methylene-protons, to those of inner part of the polymer chain, and also to those of the structural unit next to or near to the terminal structure of the polymer chain. For example, using authentic sample (Spectrum (1)), methylene proton signals were observed at 3.0 and

Figure 1. 270 MHz ^1H and 67.94 MHz ^{13}C NMR spectrum of the D_2O solution of Ca-salt of poly(L-malic acid) at 24°C in comparison to that of Ca-ion freed poly(L-malic acid).

Figure 2. 270 MHz ^1H NMR spectra of the D_2O solutions; effect of pH on aqueous solution of Ca-salt of poly(L-malic acid) at 24°C.

Figure 3. 270 MHz ^1H NMR spectra at different stage of auto-catalytic cleavage in D_2O solution: $t = 0$ min compared with that of $t = 3$ min, $t = 60$ min, and $t = 300$ min.

3.13 ppm and the methine proton signal at 5.54 ppm. A methine signal at 4.7 ppm was assigned as the terminal methine, while in Spectrum (4) ($t = 300$ min), in addition to those from increasing amounts of resulting monomeric malic acid, signals assigned to be those of methine- and methylene-protons of the unit structure next to or near to the terminal structure as defined above.

In Spectrum (3) obtained after 60 min cleavage at 100°C, the fine structure became more complex, but after comparing the spectrum with the others, assignment was made as follows: the signal intensity of both methine (5.5 ppm) and methylene (3.13 and 3.15 ppm)

Figure 4. 67.94 MHz ^{13}C NMR spectra at higher magnetic field at different stages of auto-catalytic cleavage of poly(L-malic acid) in D_2O solution: $t=0$ min compared with that of $t=30$ min, $t=60$ min, and $t=300$ min.

unit located at the inner part of the polymer chain decreased and the intensities of both methine (4.6 ppm) and methylene (2.8 ppm) protons of resulting monomeric malic acid increased. Remarkably, new signals at 3.0 and 4.7 ppm were observed, and the signals were assigned to the oligomer of malic acid produced in the cleavage reaction.

In parallel to the 270 MHz ^1H NMR spectra, 67.92 MHz ^{13}C NMR spectra were obtained on the same sample solution during the course of auto-catalytic cleavage as shown in Figure 4. The signals appearing at lower magnetic field (assigned to carbon atoms of C=O units) were complicated at the first stage of the cleavage reaction as shown in Figure 6-(2). However, the signals became simple after 300 min, giving two strong peaks due to monomeric malic acid and four peaks due to dimeric malic acid as shown in Figure 6-(3). On the basis of this assignment, the fine structure of the ^{13}C NMR spectrum at higher magnetic fields were assigned as shown in Figure 4-(4).

Table II compares the chemical shifts of these ^{13}C NMR spectra of monomeric- and dimeric-L-malic acid.

In Figures 4-(1) and -(2), the main peak at 38.40 ppm is assigned to the carbon atom of the methylene unit located at the inner part of the polymer chain and the peak at 41.25 ppm to the terminal methylene carbon. Similarly the main peak centered at 71.96 ppm is assigned to those carbon of methine unit located at the inner part of the polymer chain and the peak at 69.57 ppm is assigned to the terminal methine carbon. In contrast to those reported in previous papers,²⁻⁴ the signal assigned to the terminal structure of the authentic polymer was significant as compared to those of carbon atoms located at the inner part of the polymer chain. This means that the population of the terminal unit structure per polymer molecule is remarkably higher than those of precedents. Based on this fact, the primary structure of this poly(β -L-malic acid) was

Table II. ^{13}C NMR assignments of monomeric- and dimeric-L-malic acid in D_2O

	Chemical shifts/ppm	
	Methylene	Methine
Monomer	41.40	69.74
Dimer	38.59, 41.25	69.56, 72.29

Figure 5. The primary structure of poly(β -L-malic acid) produced by *Aureobasidium* sp. A-91.

elucidated as not linear chain polymer but with branched chains as shown in Figure 5.

As it has already been shown in analysis of the fine structure of the ^1H NMR spectra, the main peaks observed at 173.0, 174.1, 174.8, and 179.0 ppm of Spectrum (1) (Figure 6) are assigned to that of the carbonyl carbons while the two peaks at 177.4 and 179.4 ppm of Spectrum (3) are assigned to those of resulting monomeric malic acid as indicated respectively. The fine structure in Spectrum (2) was also analyzed as indicated by comparing individual signals on each spectra. In other words, Spectrum (3) indicates that the major product after 300 min heating at 100°C is monomeric L-malic acid with a minor amount of oligomer (mainly dimer of β -L-malic acid).

Thermal Properties of the Solid State Poly(β -L-malic acid)

Since this polymer is quite hygroscopic, all samples were carefully handled and sealed into aluminium pans under argon gas. It is seen in Figure 7 that neither T_g nor T_m of the solid state polymer were detected below the decomposition temperature starting at about 185°C as a distinct endothermic reaction.

Figure 6. 67.94 MHz ^{13}C NMR spectra at lower magnetic field at different stages of auto-catalytic cleavage of poly(L-malic acid): $t=0$ min compared with that of $t=60$ min and $t=300$ min.

Confirmation of Optical Purity of the Cleavage Products

The optical purity of the cleavage products as L-malic acid was assayed by an enzymatic titration using a pair of diagnostic kits for both of possible optical isomers as L-malic acid and D-malic acid. It was confirmed that both auto-catalytic cleavage and alkaline hydrolysis reaction of the poly(β -L-malic acid) gave only L-malic acid as the decomposition products and that racemization did not take

place during the course of decomposition.

CONCLUSION

Taking into account of the absence of change in methylene chemical shifts in the ^1H NMR spectra, with the significant conformational differences of the polymer chains of Ca-ion bound and Ca-ion freed poly(L-malic acid) respectively, the primary structure was deduced as poly(β -L-malic acid). Assignments

Figure 7. DSC of the solid state poly(β -L-malic acid).

of fine structures of both ^1H and ^{13}C NMR spectra could be attained comparing the spectrum recorded at different stage of cleavage *via* auto-catalytic hydrolysis of the polymer in aqueous solution. The polymer is highly hygroscopic and auto-catalytically cleaves in aqueous solution. The auto-catalytic cleavage reaction could be accelerated on heating the aqueous solution, for example, after 300 min heating at 100°C yielded L-malic acid as 98% of the polymer.

Detailed analysis of ^{13}C NMR spectra enables us to confirm the primary structure as being composed of not only linear chains but also polymer with branched chains. DSC on the solid state polymer shows neither glass transition temperature nor melting temperature below the decomposition temperature which starts at 185°C accompanied with endothermic reactions.

Enzymatic titration using a diagnostic kits applied for the cleaved products confirmed that racemization does not take place during the course of decomposition of the poly(β -L-malic acid) *via* hydrolytic cleavages either auto-catalytic or alkaline hydrolysis.

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