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Reactive oxygen species scavenging activity of aminoderivatized chitosan with different degree of deacetylation

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Abstract—Chitosans with different degree of deacetylation were prepared from crab shell chitin in the presence of alkali. Aminoderivatized chitosan derivatives were prepared in addition of amino functional groups at a hydroxyl site in the chitosan backbone. Six kinds of aminoderivatized chitosan such as aminoethyl-chitosan (AEC90), dimethylaminoethyl-chitosan (DMAEC90), and diethylaminoethyl-chitosan (DEAEC90), which were prepared from 90% deacetylated chitosan, and AEC50, DMAEC50 and DEAEC50, which were prepared from 50% deacetylated chitosan, were prepared and their reactive oxygen species (ROS) scavenging activities were investigated against hydroxyl radical, superoxide anion radical and hydrogen peroxide. The electron spin resonance (ESR) spectrum revealed that AEC90 showed the highest scavenging effects against hydroxyl and superoxide anion radical, the effects were 91.67% and 65.34% at 0.25 and 5 mg/mL, respectively. For hydrogen peroxide scavenging effect, DEAEC90 exhibited the strongest activity. These results suggest that the scavenging effect depends on their degree of deacetylation and substituted group.

1. Introduction

Free radicals and reactive oxygen species (ROS) play an important physiological role in many diseases such as cancer,[1,2] gastric ulcers,[3,4] Alzheimer's, arthritis, and ischemic reperfusion.[5] ROS, entities containing one or more reactive oxygen atoms including hydroxyl radical (·OH), superoxide anion radical $O_2^{\cdot -}$, and hydrogen peroxide (H_2O_2), are an unavoidable consequence in aerobic organisms during respiration. These radicals are very unstable and react rapidly with the other groups or substances in the body, leading to cell or tissue injury. Free radical and ROS scavenger is a preventive antioxidant. Antioxidants are substances that delay or prevent the oxidation of cellular oxidizable substrates. They exert their abilities by scavenging free radical and ROS, preventing the generation of free radical and ROS, or activating a battery of detoxifying proteins. Therefore, the role of antioxidants has received increased attention during the past decade.

Chitosan, which is a copolymer consisting of β-(1 → 4)-2-acetamido-D-glucose and β-(1 → 4)-2-amino-D-glucose units, is derived from chitin by deacetylation in the presence of alkali. Chitosan exhibits a wide variety of physiological activities such as antitumor activity,[6] immuno-stimulating effect,[7] antimicrobial effect,[8] and cholesterol-reducing effect.[9] Although chitosan has very strong functional properties in many areas, the water-insoluble property of chitosan is disadvantageous for its wide application. In the research field of chitosan, therefore, chitosan derivatives with water-soluble and functional property have been developing for food additives and new drug candidates. Most biological activities of chitosans were attributed to their free amino group at C-2 position. Therefore, in the present study, we modified C-6 position of chitosan with different degree of deacetylation and investigated ROS scavenging effects of water-soluble chitosan derivatives.

2. Results and discussion

In the present study, we synthesized water-soluble chitosan derivatives with different degree of deacetylation by grafting aminofunctionality onto chitosan at C-6 position (Scheme 1) and their ROS scavenging effects were investigated in a number of model systems. Among the

Keywords: Aminoderivatized chitosan; Electron spin resonance; Free radical; Antioxidant.
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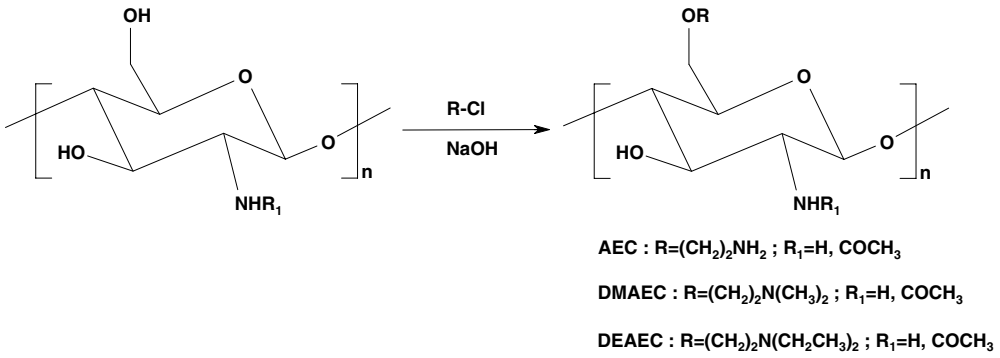

Scheme 1. Synthesis pathway of aminoderivatized chitosans.

ROS, hydroxyl radical showed the strongest chemical activity, which can easily react with amino acids, DNA and membrane components. Hydroxyl radical scavenging activities of chitosan derivatives are shown in Figure 1. Among the results recorded, AEC90 showed the highest scavenging activity, the effect was 91.67% at the concentration of 0.25 mg/mL. The scavenging effect on hydroxyl radical was in the order of AEC90 > DMAEC90 > DEAEC90, and activities were dose-dependent. Chitosan derivatives also suppressed superoxide anion radicals generated from riboflavin–EDTA system by irradiation. Superoxide radical is known to be very harmful to cellular components as a precursor of more reactive oxidative species, such as single oxygen and hydroxyl radicals. Scavenging effect of AEC90 was 65.34% at the concentration of 5 mg/mL (Fig. 2). Other derivatives showed the weak scavenging effect at the same concentration. Hydrogen peroxide, a reactive non-radical, is very important as it can penetrate biological membranes. Although H_2O_2 itself is not very reactive, it may convert into more reactive species such as singlet oxygen and hydroxyl radicals. Hydrogen peroxide scavenging activities of chitosan derivatives are shown in Figure 3. To the contrary scavenging pattern of hydroxyl

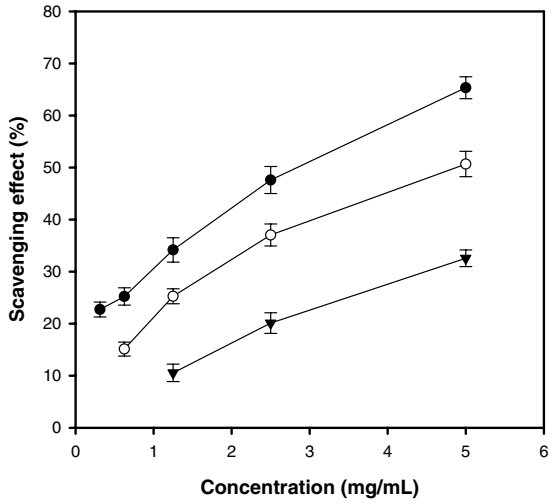

Figure 2. Superoxide anion radical scavenging effects of chitosan derivatives derived from 90% deacetylated chitosan at various concentrations. Scavenging effect was calculated by relative ESR signal intensity compared with control ESR spectrum. AEC: (–●–), DMAEC: (–○–), and DEAEC: (–▼–). Values represent means ± SE (n = 3).

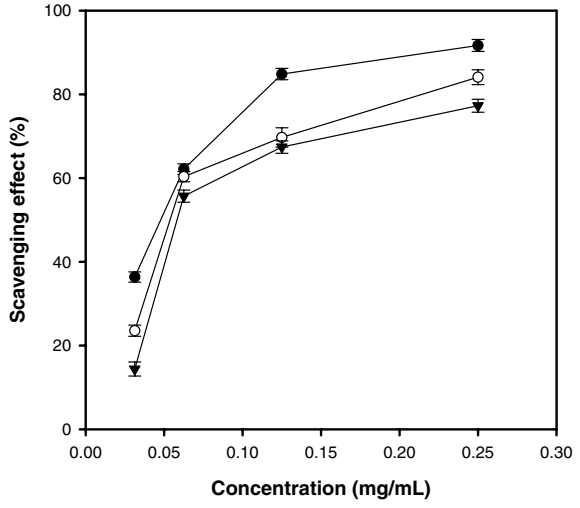

Figure 1. Hydroxyl radical scavenging effects of chitosan derivatives derived from 90% deacetylated chitosan at various concentrations. Scavenging effect was calculated by relative ESR signal intensity compared with control ESR spectrum. AEC: (–●–), DMAEC: (–○–), and DEAEC: (–▼–). Values represent means ± SE (n = 3).

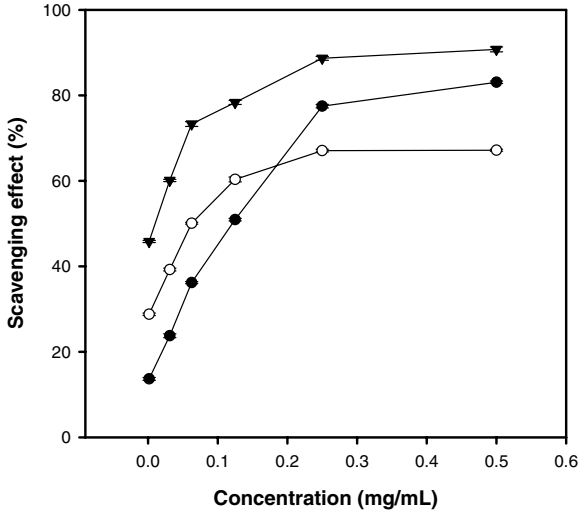

Figure 3. Hydrogen peroxide scavenging effects of chitosan derivatives derived from 90% deacetylated chitosan at various concentrations. AEC: (–●–), DMAEC: (–○–), and DEAEC: (–▼–). Values represent means ± SE (n = 3).

radical and superoxide anion radical, DEAEC90 showed the strongest scavenging activity on H_2O_2, and the scavenging activity was 88.66% at 0.25 mg/mL. Figure 4 depicts the hydroxyl radical scavenging effects of chitosan derivatives prepared from 50% deacetylated chitosan. AEC50 also quenched on hydroxyl radical up to 85% at 0.25 mg/mL, and the scavenging effect was in the order of AEC50 > DMAEC50 > DEAEC50 and the activities were dose-dependent. AEC50 also suppressed superoxide anion radical up to 58.81% at 5 mg/mL, and the scavenging effect was in a dose-dependent manner (Fig. 5). Figure 6 shows the hydrogen peroxide scavenging activities of 50% deacetylated chitosan derivatives and the scavenging pattern observed was the same as that of 90% chitosan derivatives. We used vitamin C as positive control, and the results showed that vitamin C had higher scavenging activities than that of chitosan derivatives (Fig. 7).

Recently, the antioxidant activity of chtiosan and its derivatives has been attracting the most attention. Xie et al.[10] reported that water-soluble chitosan derivatives were prepared by graft copolymerization of maleic acid sodium onto hydroxypropyl chitosan and carboxymethyl chitosan sodium, and their scavenging activities against hydroxyl radical were investigated by chemiluminescence technique. They exhibit IC_{50} values ranging from 246 to 498 μg/mL, which should be attributed to their different contents of hydroxyl and amino groups and different substituting groups. Sun et al.[11] prepared the same derivatives, and their superoxide anion scavenging activity was evaluated in a luminal-enhanced autoxidation of pyrogallol by chemiluminescence technique. They have similar IC_{50} values as vitamin C and superoxide dismutase (SOD), and IC_{50} values were ranging from 243 to 308 μg/mL. Xing et al.[12] proved the relevance of molecular weight of chitosan and its derivatives to their antioxidant activities. The antioxidant activities against hydroxyl and superoxide anion radical showed that low molecular weight chitosan exhibited the higher scavenging effects than that of high molecular weight chitosan. Moreover, low molecular chitosan sulfate had more effective scavenging activity on hydroxyl and superoxide anion radical than that of low molecular weight chitosan and high molecular weight chitosan sulfate. In this study, hydroxyl radical scavenging activities of chitosan derivatives are higher than that of Xie et al.'s result, but superoxide anion radical scavenging activities were lower than that of Sun et al.'s report and Xing et al.'s result.

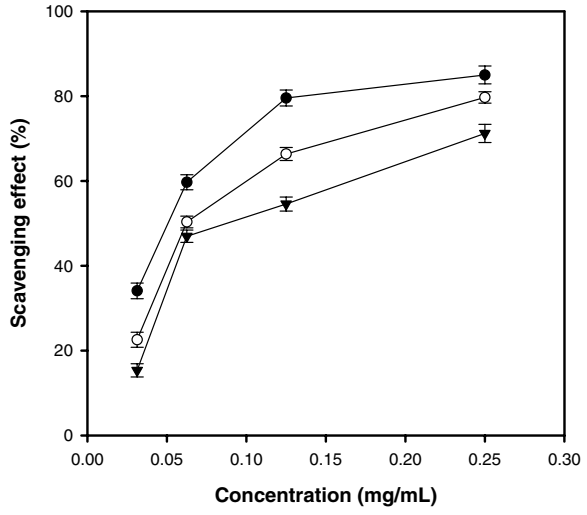

Figure 4. Hydroxyl radical scavenging effects of chitosan derivatives derived from 50% deacetylated chitosan at various concentrations. Scavenging effect was calculated by relative ESR signal intensity compared with control ESR spectrum. AEC: (–●–), DMAEC: (–○–), and DEAEC: (–▼–). Values represent means ± SE (n = 3).

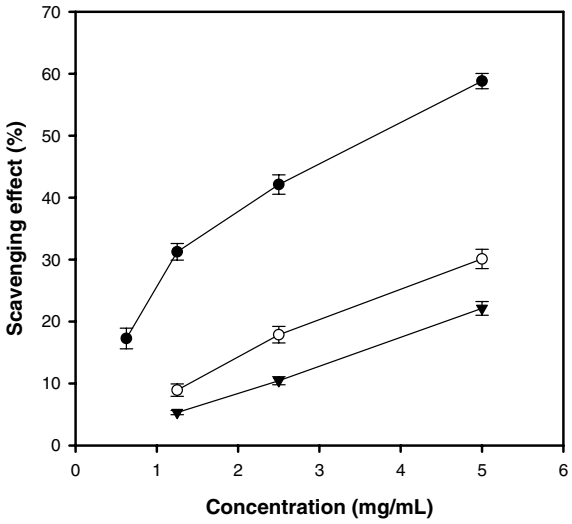

Figure 5. Superoxide anion radical scavenging effects of chitosan derivatives derived from 50% deacetylated chitosan at various concentrations. Scavenging effect was calculated by relative ESR signal intensity compared with control ESR spectrum. AEC: (–●–), DMAEC: (–○–), and DEAEC: (–▼–). Values represent means ± SE (n = 3).

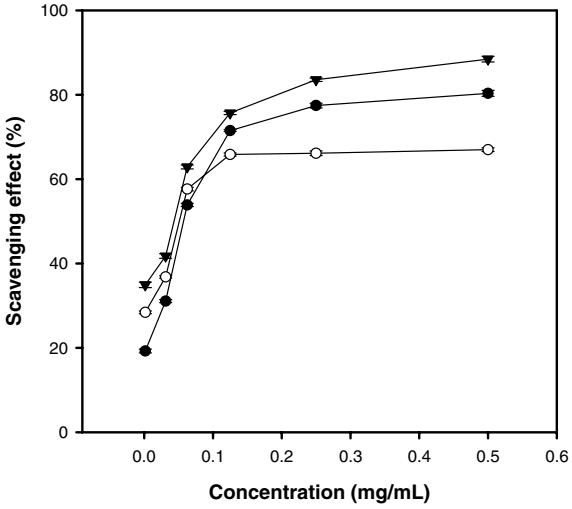

Figure 6. Hydrogen peroxide scavenging effects of chitosan derivatives derived from 50% deacetylated chitosan at various concentrations. AEC: (–●–), DMAEC: (–○–), and DEAEC: (–▼–). Values represent means ± SE (n = 3).

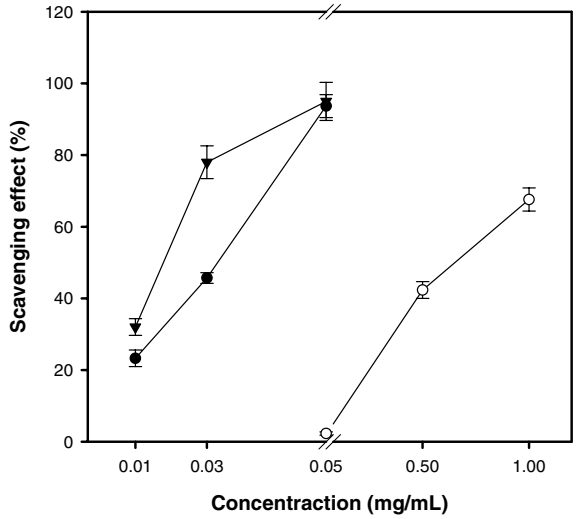

Figure 7. Reactive oxygen species scavenging effects of vitamin C as positive control. Hydroxyl radical: (–●–), superoxide anion radical: (–○–), and hydrogen peroxide: (–▼–). Values represent means ± SE ($n = 3$).

In our previous report, we prepared three kinds of chitosans with different degree of deacetylation from crab shell chitin, and investigated their free radical scavenging activities on DPPH, hydroxyl, superoxide, and alkyl radical using ESR.[13] The results showed that 90% deacetylated chitosan exhibited the strongest free radical scavenging effects, and the effect was dependent on their degree of deacetylation. Chen et al.[14] investigated the antioxidant of chitobiose and chitotriose using the inhibition of H_2O_2-induced hydroxylation of benzoate and free radical scavenging effects, and the results revealed that chitobiose is more effective than chitotriose. Our laboratory also prepared the five chitooligosaccharides (COSs) with different molecular weights and investigated the free radical scavenging activities, and COSs (MW, 3000–5000 Da) exhibited above 95% scavenging effect on DPPH radical at 0.05% concentration.[15] In addition, COSs (MW, 3000–5000 Da) showed above 95% scavenging effect on hydroxyl radical at a dosage of 0.25 mg/mL. Comparing with these data, in the present study, the aminoethylated chitosan derivatives showed higher free radical scavenging effects as that of chitosan and similar effects than that of chitosan oligomers. We also used vitamin C as positive control and tested on free radical scavenging effects. The radical scavenging activity of vitamin C was a higher than those of derivatives. Vitamin C was actually a potent hydroxyl and carbon-centered radical scavenger, but simultaneously formed its own radical, ascorbyl radical.[16] Podmore et al.[17] reported that an ascorbyl radical might cause oxidative reactions on other materials, and that vitamin C had a pro-oxidant activity.

Several researchers suggested the scavenging mechanism of chitosan on free radicals that hydroxyl and superoxide anion radicals can react with active hydrogen atoms in chitosan to form a most stable macromolecule radical. In the structure of chitosan, there are three hydrogen sources at C-2 (NH_2) and C-3, -6 (OH) positions, however, it is difficult to react with OH of C-3 position because they have steric hindrance. So, the major target of chitosan for modification is introduction on NH_2 or OH of C-2 and -6 positions. In the present study, we modified chitosan at C-6 position with different substitution groups, and evaluated their ROS scavenging activities on various radical generated systems. The results suggested that NH_2 group is major factor for free radical scavenging activities because AEC90 showed stronger ROS scavenging activities than that of AEC50, and also AEC90 was more potent than DMAEC90 and DEAEC90, especially against hydroxyl and superoxide anion radicals. The same pattern was observed in chitosan derivatives derived from 50% deacetylated chitosan. However, the scavenging activities of chitosan derivatives were not much improved than that of chitosan in spite of introduction of amino group on C-6 position.

3. Conclusions

In this paper, we synthesized water-soluble chitosan derivatives, and their ROS scavenging effects were investigated in a number of model systems. Among the derivatives, AEC90 was more potent ROS scavenger than other derivatives, and major factor affecting ROS scavenging activity was free amino group. These in vitro results suggest the possibility that AEC90 could be used as ingredient in health or functional food.

4. Materials and methods

4.1. Materials

Chitin prepared from crab shells was donated by Kitto Life Co. (Seoul, Korea). For the preparation of chitosan derivatives, 2-chloroethylamino hydrochloride was purchased from Fluka, 2-(dimethylamino)ethylchloride hydrochloride and 2-(diethylamino)ethylchloride hydrochloride, 2,2-azinobis(3-ethylbenzthiazolin)-6-sulfonic acid (ATS), peroxidase, riboflavin, ethylenediamine tetraacetic acid (EDTA), $FeSO_4$, hydrogen peroxide, and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were of the highest grade available commercially.

4.2. Preparation of chitosans with different degree of deacetylation

Partially deacetylated chitosans were prepared and the degree of deacetylation was determined by FT-IR and titration method according to our report.[18] The degree of deacetylation designated as 90% and 50%. The average molecular weights of the chitosans were 3.1×10^5 as determined by viscosimetry.[19] After the reaction, chitosan samples were washed thoroughly with distilled water and freeze-dried.

4.3. Synthesis of aminoderivatized chitosans

Aminoderivatized chitosans were prepared according to our previous method (Scheme 1).[20] Aqueous 3.0 M

(15 mL) aminoalkyl hydrochloride was added to chitosan (0.30 g) with stirring at 65 °C. NaOH of 3.0 M (15 mL) was added to the reaction mixture dropwise, and continuously stirred for 18 h. Subsequently, the reaction mixture was acidified with HCl and dialyzed against water for 2 days. The product was freeze-dried to give the aminoderivatized chitosan (AEC90: 0.412 g, DMAEC90: 0.393 g, DEAEC90: 0.489 g, AEC50: 0.401 g, DMAEC50: 0.376 g, and DEAEC50: 0.476 g) and was found to dissolve well in water and all pH range. Water-soluble chitosan derivatives were designated as aminoethyl-chitosan (AEC90), dimethylaminoethyl-chitosan (DMAEC90), and diethylaminoethyl-chitosan (DEAEC90) prepared from 90% deacetylated chitosan, and AEC50, DMAEC50, and DEAEC50 prepared from 50% deacetylated chitosan. FT-IR spectra showed the new peak at 2965 cm^{-1} due to C–H stretching of substituted groups. The ^1H NMR spectra of DEAEC90 in D_2O showed peak at 1.30 ppm for the methyl, at 3.28 ppm for the methylene protons of the DEAE group, and between 1.5 and 1.6 ppm for the methyl protons of the protonated DEAE groups. In the same manner, the peak observed between 2.9 and 3.0 ppm for methyl and methylene protons of the DMAE group, and the peak at 2.9 ppm for methylene protons of the AE group were also observed.[20] AEC50, DMAEC50, and DEAEC50 were characterized in the same manner.

4.4. Hydroxyl radical scavenging assay

Hydroxyl radicals were generated by iron-catalyzed Haber–Weiss reaction (Fenton driven Haber–Weiss reaction) and the generated hydroxyl radicals rapidly reacted with nitrone spin trap DMPO.[21] The resultant DMPO-OH adduct was detectable with an ESR spectrometer. Derivatives (0.2 mL) with various concentrations were mixed with 0.3 M DMPO (0.2 mL), 10 mM $FeSO_4$ (0.2 mL), and 10 mM H_2O_2 (0.2 mL) in a phosphate buffer solution (pH 7.2), and then transferred into a 100 μL quartz capillary tube. After 2.5 min, the ESR spectrum was recorded using an ESR spectrometer. Measurement conditions: magnetic field, 336.5 ± 5 mT; power, 1 mW; modulation frequency, 9.41 GHz; amplitude, 1×200; sweep time, 4 min.

4.5. Superoxide radical scavenging activity

Superoxide anion radicals were generated by UV irradiation of a riboflavin/EDTA solution.[22] The reaction mixtures containing 0.8 mM riboflavin, 1.6 mM EDTA, 800 mM DMPO, and various concentrations of derivatives were irradiated for 1 min under UV lamp at 365 nm. The mixtures were transferred to 100 μL quartz capillary tube of the ESR spectrometer for measurement. Measurement conditions: magnetic field, 336.5 ± 5 mT; power, 10 mW; modulation frequency, 9.41 GHz; amplitude, 1×1000; sweep time, 1 min.

4.6. Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging assay was carried out according to the method of Muller.[23] Derivatives with various concentrations (100 μL) and 10 mM hydrogen peroxide (20 μL) were mixed with 0.1 M phosphate buffer (pH 6.0, 70 μL) in a 96-microwell plate and incubated at 37 °C for 5 min. Thereafter, 30 μL of freshly prepared 1.25 mM ATS and 30 μL of peroxidase (1 U/mL) were mixed and incubated at 37 °C for 10 min and the absorbance was recorded with an ELISA reader at 405 nm.

4.7. Statistics

The data presented are means ± SE of three determinations.

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