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EFFECT OF MODIFICATION OF SOYBEAN LIPOXYGENASE-1 WITH N-BROMOSUCCINIMIDE ON LINOLEATE OXIDATION, PIGMENT BLEACHING AND CARBONYL PRODUCTION

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Key Word Index—Lipoxygenase-1, N-bromosuccinimide, pigment bleaching, carotene, chlorophyll, anaerobic bleaching, linoleate oxidation, tryptophan.

Abstract—Essential tryptophan residues were specifically modified in soybean lipoxygenase-1 by N-bromosuccinimide (NBS). Both linoleate oxidation and pigment bleaching (β-carotene or chlorophyll *a*) activities were significantly reduced with the modified enzyme under aerobic conditions. However, the effect on the reduction of linoleate oxidation was more marked. Pigment bleaching under anaerobic conditions was almost completely blocked with the modified enzyme. On the basis of spectral studies it was elucidated that soybean lipoxygenase-1 contains two essential tryptophan residues in its active site.

INTRODUCTION

Lipoxygenase (linoleate: oxygen oxidoreductase EC 1.13.11.12) is known to be involved in the *in vitro* bleaching of plant pigments (chlorophyll and carotene). The co-oxidation of carotene and chlorophyll during enzymatic fatty acid oxidation has been attributed to certain lipoxygenase isoenzymes [1, 2]. In our laboratories, we have been investigating *in vitro* and *in vivo* pigment bleaching by lipoxygenases [3]. Recently we have shown that soybean lipoxygenase type-1 is a powerful carotene and chlorophyll bleacher under anaerobic conditions [4]. Moreover, by studying the effect of various antioxidants on the bleaching activities of this isoenzyme, we sugested that the enzyme–fatty acid complex is the main driving force in the bleaching. In order to better understand the involvement of the active site of lipoxygenase in the co-oxidation pathways, we took the approach of studying the essential amino acid residues involved in activity by chemical modification. In a previous study, we have shown that specifically modifying –SH residues in the active site of soybean lipoxygenase type-1 on the one hand significantly inhibited linoleate oxidation, but on the other hand, strongly enhanced the pigment bleaching potential of the enzyme both under anaerobic and aerobic conditions [5]. In the present study, the implications of specifically modifying tryptophan residues in soybean lipoxygenase-1 on pigment bleaching, linoleate oxidation and carbonyl production are described.

RESULTS

Effect of NBS on linoleate oxidation and pigment bleaching under aerobic conditions

When lipoxygenase-1 was treated with NBS at pH 4.0, its linoleate oxidation capacity was markedly reduced. As can be seen from the kinetics of the reaction (Fig. 1), the modifier strongly affected the initial rate of linoleate oxidation and during the first 30 sec there was almost complete inhibition of conjugated diene formation. Under aerobic conditions, 4×10^{-4} M NBS caused almost 90% inhibition of conjugated diene formation, almost 12% inhibition of chlorophyll bleaching and about 70% inhibition of carotene bleaching. The same relative results were obtained with different enzyme concentrations.

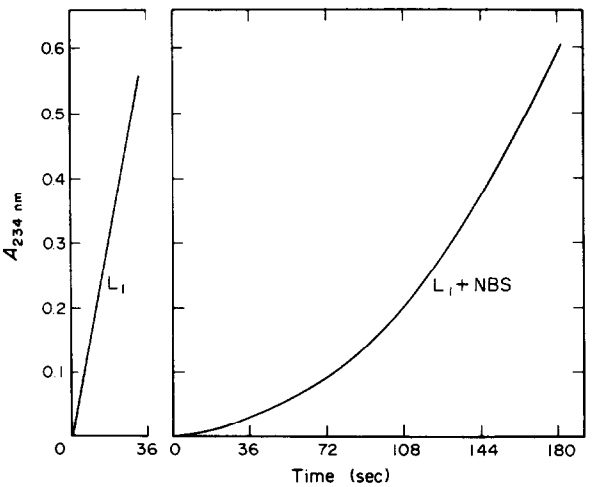

Fig. 1. Effect of modification with NBS on linoleate oxidation by soybean lipoxygenase-1. Twenty μl of NBS (0.02 M in H_2O) were added to 1 ml of enzyme (type-I)/sample (0.3 mg/ml) in 0.2 M sodium acetate buffer, pH 4.0. After incubation for 20 min, 15 μg of modified enzyme and control sample were assayed for linoleate oxidation, as described in Experimental.

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Effect of NBS on pigment bleaching, linoleate oxidation and carbonyl production under anaerobic conditions

As pointed out previously [4], soybean lipoxygenase type-1 is a powerful pigment bleaching factor under anaerobic conditions. From the data shown in Table 1, it can be seen that while the unmodified enzyme strongly bleached carotene under anaerobic conditions, the modified enzyme lost most of this capability and its co-oxidizing activity was 90% reduced. The effect of modification on the bleaching of carotene by soybean lipoxygenase type-1 under aerobic conditions was 10-fold lower when compared to the activity under anaerobic conditions (Table 1). Moreover, only the unmodified enzyme exhibited high carbonyl production (A_{285}) under anaerobic conditions, while the modified enzyme did not cause any increase in absorbance at 285 nm under similar conditions. Linoleate oxidation in the presence of carotene both under O_2 or air conditions was almost completely blocked when the enzyme was modified with 1×10^{-4} M NBS (Table 1).

The effect of NBS modification (1×10^{-4} M) and (5×10^{-5} M) on the bleaching of chlorophyll by soybean type-1 enzyme under anaerobic and aerobic conditions is presented in Table 2. In the presence of chlorophyll, linoleate oxidation under aerobic conditions was 55% reduced when the enzyme was modified with 5×10^{-5} M NBS and 90% when NBS was used at a concentration of 1×10^{-4} M. Chlorophyll bleaching and carbonyl production (A_{285}) under anaerobic conditions were also strongly inhibited as a result of this modification. Although the modified enzyme was slightly more active in bleaching chlorophyll under aerobic conditions, this activity, in general, was very low compared to the bleaching activity obtained under anaerobic conditions (Table 2). When the soybean lipoxygenase type-1 was modified with a lower concentration of NBS (5×10^{-5} M), linoleate, oxidation in the presence of chlorophyll was 50% inhibited. Similar patterns of inhibition were found with carotene bleaching and carbonyl production (A_{285}) under anaerobic conditions.

Effect of modification with NBS on the spectrum of lipoxygenase-1

In Fig. 2 (a and b), the strong effect of NBS modification on the UV spectrum of the enzyme is shown. As a result of the modification, the absorbance at 280 nm, mainly due to the presence of tryptophan residues in the enzyme protein, is significantly reduced. Under the conditions described in Fig. 2(a), the enzyme completely lost its ability to oxidize linoleate as a result of the modification, while under the conditions described in Fig. 2(b), linoleate oxidation by the enzyme was 88% inhibited. Based on the method described by Spande and Witkop [6], it can be calculated that when the enzyme was completely inhibited, 1.9 residues of tryptophan were

Table 1. Effect of modification of soybean lipoxygenase type-1 with NBS on carotene bleaching (A_{460}), carbonyl production (A_{285}) and linoleate oxidation under anaerobic and aerobic conditions

	ΔA_{460}/min			ΔA_{285}/min			ΔA_{234}/min		
Sample	Anaerobic (Ar)	Aerobic (O_2)	Aerobic (air)	Anaerobic (Ar)	Aerobic (O_2)	Aerobic (air)	Anaerobic (Ar)	Aerobic (O_2)	Aerobic (air)
Control	0.115	0.012	0.012	0.089	0	0	−0.05	3.7	2.6
Modified	0.014	0.006	0.008	0	0	0	0.01	0.1	0.1

Lipoxygenase (0.3 mg/ml [Sigma, type V, 735000 units/mg protein]) at pH 4.0 was incubated for 20 min with 1×10^{-4} M NBS and 25 μl (7.5 μg) were added to the reaction mixture which contained 200 μM linoleate and 3.7 μM β-carotene at pH 9.0.

Table 2. Effect of modification of soybean lipoxygenase type-1 with NBS on chlorophyll bleaching (A_{663}), carbonyl production (A_{285}) and linoleate oxidation (A_{234}) under anaerobic and aerobic conditions

	ΔA_{663}/min		ΔA_{285}/min		ΔA_{234}/min	
Sample	Anaerobic (Ar)	Aerobic (air)	Anaerobic (Ar)	Aerobic (air)	Anaerobic (Ar)	Aerobic (air)
			5×10^{-5} M NBS			
Control	0.040	0.003	0.050	0	−0.035	3.70
Modified	0.021	0.005	0.015	0	−0.015	1.65
			1×10^{-4} M NBS			
Control	0.115	0.001	0.093	0	−0.045	4.0
Modified	0.006	0.002	0	0	0.090	0.45

All conditions were as in Table 1 except that instead of carotene, 5.6 μM chlorophyll *a* were added to the reaction mixture.

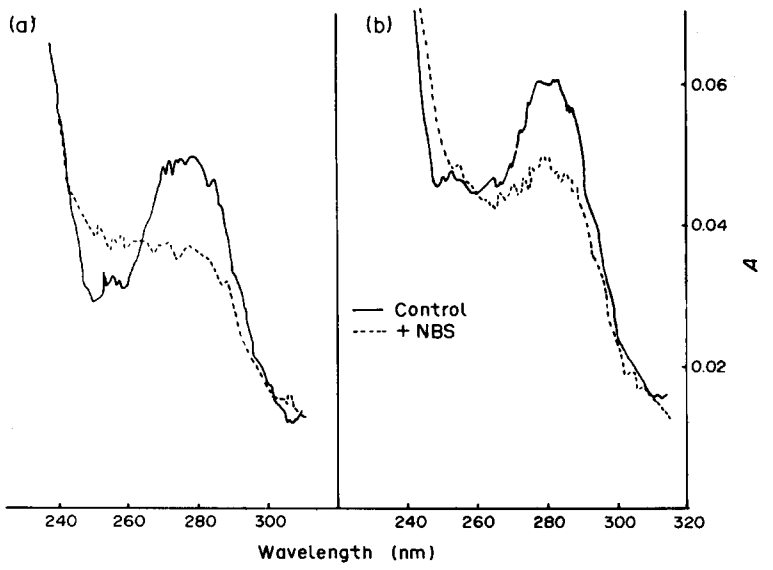

Fig. 2. UV spectra of soybean lipoxygenase-1 and NBS modified enzyme. (a) 1 μl NBS (0.02 M) were added to 0.15 mg enzyme (type-V)/ml in sodium acetate, pH 4.0. Final volume 2.5 ml. (b) 2.5 μl NBS (0.02 M) were added to 0.12 mg enzyme/ml in sodium acetate, pH 4.0. Final volume 2.5 ml.

oxidized and only 1.5 residues were oxidized when the inhibition was 88% (Fig. 2b).

DISCUSSION

Although a first indication about a possible role of tryptophan residues in the activity of soybean lipoxygenase was reported by us as early as 1971 [7], the preparation used for that study was a mixture of at least two soybean isoenzymes. Based on fluorescence perturbation experiments, Finazzi-Agro *et al.* [8] proposed that soybean lipoxygenase type-1 has a large hydrophobic active site which contains the tryptophan residues of the protein and iron. In the present study, it is clearly elucidated that tryptophan is an essential amino acid in the active site of soybean lipoxygenase type-1 and as a result of a specific modification with NBS, the linoleate-induced oxidation is lost. The data presented in Fig. 2 indicate that there are two essential tryptophan residues in the active site of soybean lipoxygenase type-1 and when both are oxidized the enzyme completely loses its ability to oxidize linoleate. As a result of the modification, the co-oxidizing activity of the enzyme is also significantly affected, and the bleaching of the pigments β-carotene and chlorophyll *a* is markedly reduced.

Two kinds of preparations of purified soybean lipoxygenase type-1 were used in this study which showed approximately the same pattern of behaviour under the conditions of the assay. One of the preparations (Sigma type-1, purified) exhibited slightly more bleaching activity under aerobic conditions although, as shown before [4] and also in this study, the bleaching of carotene and chlorophyll is generally several-fold higher under anaerobic conditions than under aerobic atmosphere.

In previous studies [5, 9], it was elucidated that when free –SH groups are modified in the active site of soybean lipoxygenase type-1, its activity is influenced in two different ways. On one hand, linoleate oxidation by the enzyme is significantly inhibited, while on the other hand, its ability to bleach β-carotene or chlorophyll *a* is markedly enhanced. The explanation for this mode of behaviour was based on the assumption that the conformational structure of the enzyme is changed as a result of the modification. This creates better conditions for a lipoxygenase–fatty acid–pigment interaction, thus leading toward an alternative sequence of reactions which result in an increased rate of pigment bleaching. However, based on the data obtained in this study, it may be assumed that the conformational changes in the active site of lipoxygenase type-1 as a result of tryptophan oxidation are completely different from those obtained when –SH groups are modified. It seems that tryptophan residues are essential for both linoleate oxidation and pigment bleaching activities and any change in these residues immediately inhibits these activities.

EXPERIMENTAL

Enzyme source and purification. Two different soybean lipoxygenase-1 preparations (Sigma Chemical Co., St. Louis, MO) were used for the experiments: type-1, 125 000 units/mg protein, and type-V, purified by affinity chromatography, 735 000 units/mg protein. Further purification of the type-1 lipoxygenase was done by the method of Finazzi-Agro *et al.* [8]. The enzyme preparation used in each experiment is given in the tables.

Modification procedure. All modifications of soybean lipoxygenase-1 were done at room temp (25°). In general, various amounts (μl) of NBS (0.02 M in H_2O) were added to an enzyme sample (0.3 mg/ml) in 0.2 M NaOAc buffer, pH 4.0. After incubation for at least 20 min, the modified enzyme was kept at 4°. Control enzyme (unmodified) samples were treated similarly, except that H_2O was added instead of NBS. The modified and unmodified (control) enzyme could be held for at least 3 hr at 4° without any change in enzymatic activity.

Assay procedures. Anaerobic conditions were obtained by saturating all the solutions with Ar, as well as the reaction mixture in the cuvette before addition of the enzyme. When aerobic

conditions were used, the reaction mixture was flushed with O_2. Air saturation conditions were achieved by using solns equilibrated with air at room temp.

Conjugated diene formation with linoleate as substrate was followed at 234 nm according to Ben-Aziz *et al.* [10]. Carotene and chlorophyll bleaching activities were determined spectrophotometrically at 460 or 663 nm, respectively, according to Reynolds and Klein [11]. Carbonyl production in the presence of the pigments was measured as the change in absorbance at 285 nm during the course of the reaction. Concentrations of reactants used are shown in the tables. Appropriate blanks were run for each reaction. Activities are expressed as the change in absorbance (A) at the specified wavelength.

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