Effect of Succinylation on Molecular and Functional Properties of Soluble Tobacco Leaf Proteins†

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Fraction 1 protein (F-1-p) and fraction 2 protein (F-2-p) from tobacco leaf were progressively modified with succinic anhydride. At a 1:1 ratio, succinylation reached 80% and 98% nucleophilic groups of the respective proteins. It dissociated F-1-p into subunits with increased molecular weight. The latter phenomenon is also evident for F-2-p. Succinylation doubled the bulk volume of F-1-p, whereas F-2-p expanded 70% followed by a decline due to extensive modification. Although the solubility index was improved, foaming capacity and stability changed little for F-1-p and decreased slightly for F-2-p. Increase in water absorption and fat-binding capacity is positively correlated with the extent of modification. Succinylation did not alter the emulsifying activity index of F-1-p but enhanced the emulsifying capacity by 56%. The latter was doubled for the modified F-2-p which also gained 3-fold in emulsifying activity index. The present study showed the differential response in functional improvement of leaf protein fractions modified by succinylation.

INTRODUCTION

Protein in green vegetation is the most abundant source of protein on earth. It has been estimated that through solar radiation 1 ha of land in the nonwoodland ecosystem could produce approximately 9×10^3 kg of crude protein of plant origin per annum (Phillipson, 1973). When leafy tissue is pulverized and juice filtered off from fibrous residue, the filtrate yields a green sediment containing insoluble protein of cell membrane for animal feed and soluble protein in its supernatant which can be coagulated by heat and/or acid treatment (Telek and Graham, 1983). The coagulant of soluble leaf protein is a mixture of fraction 1 protein (F-1-p) and fraction 2 protein (F-2-p), terms coined by Wildman and Bonner (1947). F-1-p is ribulose 1,5-bisphosphate carboxylase/oxygenase catalyzing photosynthesis and photorespiration in chloroplasts (Kung, 1976), whereas F-2-p is the remaining cytoplasmic proteins. This protein mixture from many plant species has a balanced amino acid composition suitable as dietary protein to supplement nutritional needs for people of less developed countries (Telek and Graham, 1983). However, heat and acid treatments cause protein denaturation and insolubility. Insoluble protein usually exhibits minimal functional properties that are essential in food formulation.

Intentional chemical modification of food protein and novel proteins has been reported for improvement of solubility and functionality (Feeney, 1986). Succinic anhydride has been frequently used to improve protein solubility and dispersion property. Succinylation increased water absorption and emulsifying capacity of wheat gluten (Barber and Warthesen, 1982) and emulsifying and foaming properties of soy protein (Franzen and Kinsella, 1976a) and leaf protein (Franzen and Kinsella, 1976b). For soy protein, the improved functionality is in part associated with the enhanced hydrophobicity and viscosity of soy glycinin (Kim and Kinsella, 1986, 1987). In contrast, the results of succinylated leaf protein did not distinguish the effect of chemical modification on the individual leaf protein fractions. One would expect that upon succinylation the interaction of dissociated subunits and unfolded polypeptides of F-1-p would be different in the presence or absence of F-2-p and vice versa.

The recent advancement in leaf protein process achieved a complete separation of F-1-p and F-2-p on an industrial scale, and yet F-1-p was isolated in nondenatured state (Wildman, 1983; Sheen, 1986). The freeze-dried powder of both leaf protein fractions showed exceptional functionality except for poor solubility under acidic conditions (Sheen, 1991). Increase in solubility of F-1-p was attained by partial degradation with some chemical and enzymatic means, but this resulted in a decrease of certain functional attributes (Sheen and Sheen, 1988). How succinylation affects the functional properties in general and solubility in particular of leaf protein fractions has not been studied. Furthermore, the extent of succinylation for the available amino and/or nucleophilic groups of leaf protein fractions may modify functionality in a different manner. The present study was therefore undertaken to evaluate the effect of progressive succinylation on the molecular and functional modification of tobacco F-1-p and F-2-p.

MATERIALS AND METHODS

Leaf Protein Preparation. Tobacco (*Nicotiana tabacum* L., cultivar Ky 14) was grown in 8-in. pots in the greenhouse. Growth conditions were 14-h photoperiod, 28 °C for daytime and 20 °C at night, and nutritional solution was added three times per week. Plants at 14–16-leaf stage were harvested in three groups, about 18–20 plants per group. Leaves of each group were separately processed by a previously described procedure to isolate crystalline F-1-p and acid-precipitated F-2-p (Sheen, 1986). Protein crystals and precipitates were freeze-dried and stored as dry powder prior to chemical treatment and analysis.

Succinylation of Leaf Protein. The method of Franzen and Kinsella (1976b) for protein succinylation was followed with modifications. Because of precipitation in 0.1 M phosphate buffer, pH 8.2, crystalline F-1-p was solubilized in distilled water instead, and the protein solution in concentration of 3 g/60 mL was adjusted to pH 8.1 with 0.1 N NaOH. Succinic anhydride in amounts of 0.3, 0.6, 1.5, and 3.0 g, which give ratios of succinic anhydride to protein of 0.1, 0.2, 0.5, and 1.0, was added in small increments to the protein solution while the solution was continuously stirred and maintained above pH 8.1 by addition of 3 N NaOH at 25 °C. Succinylation of F-2-p was similarly processed. Protein solution at pH 8.1 without succinic anhydride was the control. All treatments were dialyzed in membrane tub-

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ings of molecular weight cutoff at 3500 against distilled water for 48 h at 4 °C. The distilled water for dialysis was adjusted to pH 8 with 0.1 N NaOH and changed four times to remove excess reagent.

The extent of succinylation in terms of the percent of amino groups succinylated as determined by the reaction of free amino groups with trinitrobenzenesulfonate (TNBS) was determined according to the method of Fields (1972). Twenty milligrams of protein was solubilized in 1 mL of 0.1 M sodium borate buffer, pH 10, and 0.6 mL of water and 0.4 mL of TNBS were added in sequence. The solution was rapidly mixed and held at ambient temperature for 5 min prior to the addition of 4 mL of freshly prepared 1.5 mM sulfite solution. The absorbance of the sample was read at 420 nm against a blank identically prepared with 1 mL of water in place of TNBS.

Molecular Property. Ultraviolet (UV) absorbance and polyacrylamide gel electrophoresis (PAGE) were employed to evaluate the effect of succinylation on the molecular properties of leaf protein fractions. The UV spectra of 0.1% protein in 20 mM phosphate buffer, pH 8, were recorded in a Cary 210 spectrophotometer at 1-nm bandwidth in the 330–230-nm range with a matched pair of quartz cuvettes having 1 cm path length. Nondissociating PAGE and SDS–PAGE were performed on gel slabs with a linear gradient of 3–17.5% and 7–17.5% acrylamide, respectively. An aliquot of 300 μg of protein in the sampling buffer, pH 6.8, was applied to each lane for both F-1-p and F-2-p. Such a high concentration of protein was intentially chosen to compensate the partial solubility and poor dye binding of the succinylated F-2-p. Electrophoretic procedure and band visualization with Coomassie Brilliant Blue R-250 were identical with that described in a recent paper (Sheen, 1989).

Functional Property. The solubility index determined with the Bio-Rad protein assay method (Sheen and Sheen, 1988) appeared to be lower in proportion to the percent succinylation of the protein. This is possibly attributable to the interference of succinylation to dye binding. The gravimetric method was therefore used to determine the solubility index. Protein (0.1 g) was solubilized by vortexing in a centrifuge table with 10 mL (0.1 M) each of pH 3 and 5 citrate–phosphate buffer, pH 7.4, phosphate buffer, and pH 9 boric acid–borax buffer. After centrifugation at 10000*g* for 20 min, the insoluble sediment was resuspended in 10 mL of distilled water and centrifuged in the same manner to remove buffer salts. The insoluble sediment was then freeze-dried and weighed. Solubility index is calculated as the percent weight loss.

Foaming capacity and stability were measured according to the method of Lawhon and Cater (1971), while water absorption was determined according to the procedure of Fleming et al. (1974). The determination of fat-binding capacity was made according to the turbidimetric method of Voutsinas and Nakai (1983). The turbidimetric method developed by Pearce and Kinsella (1978) and modified by Li-Chan et al. (1984) for determination of emulsifying activity index was adapted except that the measurement at 500 nm was made with a Cary 210 spectrophotometer. Emulsifying capacity, defined as milliliters of oil per gram of protein was measured by the oil titration method with a volt–ohm meter as described by Regenstein and Regenstein (1984).

Statistics. There are 30 protein samples, 15 each for F-1-p and F-2-p. Within the protein fraction, there are five treatments with three replications as represented by three sampling groups. The data were analyzed for variance according to a randomized replicate design. Treatment means were compared with the least significant difference (lsd) at the 5% and 1% levels of probability. Statistical methods were given by Steel and Torrie (1960).

RESULTS

General Property. The percent succinylation of tobacco F-1-p and F-2-p at the ε-amino group of lysine and α-amino group of polypeptides reached more than two-thirds of the amino residues at a succinic anhydride to protein ratio of 0.1:1 (Table I). Subsequent increase in succinylation was insignificant with respect to the amount of the acylating reagent. At the 1:1 ratio, the oligomeric F-1-p with structurally compact large and small subunits had about 80% amino groups modified. In contrast, the corresponding modification of F-2-p reached 98.4%, suggesting that its heterogeneous mixture of proteins is readily accessible to succinic anhydride. The increase in percent succinylation of F-2-p appeared to coincide with the acylating agent to protein ratio at a statistically significant level.

Table I. Effect of Percent Succinylation on the Bulk Volume of Soluble Tobacco Leaf Proteins

leaf protein	ratio of succinic anhydride to protein, g/g	% succinylation	bulk volume, mL/g
F-1-p	0	0	2.1
	0.1	66.7	3.7
	0.2	74.9	3.8
	0.5	76.3	4.3
	1.0	79.8	4.6
	lsd 0.05	7.5	0.7
	lsd 0.01	10.9	1.0
F-2-p	0	0	2.1
	0.1	72.8	3.4
	0.2	78.3	3.6
	0.5	89.7	2.9
	1.0	98.4	2.8
	lsd 0.05	8.2	0.7
	lsd 0.01	11.9	1.0

Succinylation resulted in an increase in bulk volume of the leaf protein fractions (Table I). For F-1-p, the increase was positively correlated with the percent succinylation and was up to more than 100% at the high succinic anhydride to protein ratio. About a 70% increase in volume of F-2-p occurred at the 0.2:1 ratio, which was followed by a decline as more amino and/or other nucleophilic groups were acylated. In all cases, the succinylated leaf proteins are lighter in color and excellent in dispersibility and wettability. They are also odorless and tasteless as the unmodified proteins.

Molecular Modification. In comparison to native protein which has an absorbance maximum at 279 nm, the progressive succinylation caused a shift of the maximum toward shorter wavelength. This blue shift changed the maximum to 276 nm for the highly acylated F-1-p. At 280 nm, the absorbance of the modified F-1-p reduced to 92%, 87%, 85%, and 82% of that of the native protein as the extent of succinylation increased. The UV spectrum of F-2-p, either succinylated or not, showed no definitive peaks other than a continuous increase of absorbance from 330 to 230 nm. This is possibly due to soluble cellular components which formed ligands with F-2-p during extraction.

Nondissociating PAGE of tobacco F-1-p in lane 1 (Figure 1) revealed a major protein band representing the native form with molecular weight around 560 000. A minor band in low molecular weight is likely a degradation product due to the freeze-drying process rather than F-2-p contaminant. This is substantiated by the absence of the same minor bands in F-2-p on the SDS–PAGE gel (lanes 11 and 16). Succinylation partially dissociated F-1-p into a number of low molecular weight bands, some of which gained molecular weight as the extent of modification increased (lanes 2–5). This phenomenon is more clear for the banding pattern of SDS–PAGE in lanes 12–15, where both large and small subunits of F-1-p appeared in reduced mobility as a function of percent succinylation. In lanes 17–20, molecular alteration and slow band mobility of the modified F-2-p were also evident on the SDS–PAGE gel. On the nondissociating gel, the freeze-dried F-2-p smeared throughout the gel irrespective of succinylation (lanes 6–10). The possible reasons underlying the weak staining

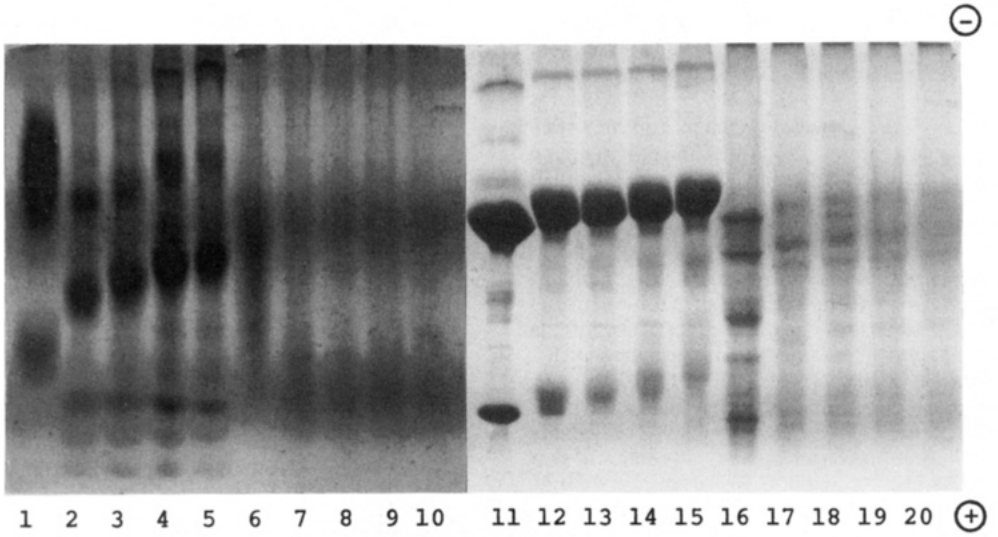

Figure 1. Gradient PAGE for the soluble tobacco leaf proteins. Lanes 1–5 and 6–10 are on nondissociating gel for F-1-p and F-2-p in the succinic anhydride to protein ratios of 0, 0.1, 0.2, 0.5, and 1.0, respectively. The same order of samples on SDS dissociating gel applies to lanes 11–15 for F-1-p and lanes 16–20 for F-2-p.

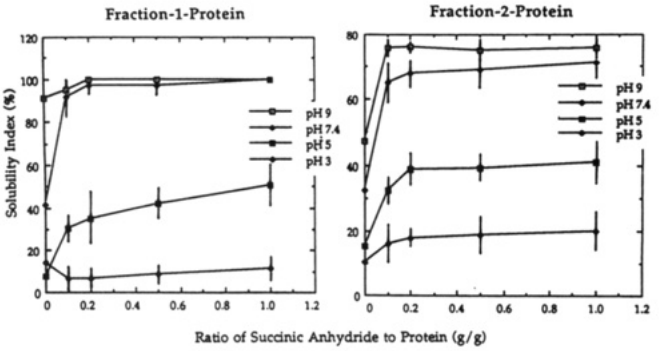

Figure 2. Change of solubility index for F-1-p and F-2-p progressively modified with succinic anhydride in 0.1 M each of citrate–phosphate buffer, pH 3 and 5, phosphate buffer, pH 7.4, and boric acid–borax buffer, pH 9. The vertical bar denotes the standard error of the mean.

of the succinylated F-2-p with Coomassie Brilliant Blue will be discussed later.

Functional Property. Excluding F-1-p in pH 3 solution, succinylation improved solubility index in all treatments (Figure 2). Improvement was significant even at the 0.1:1 ratio of succinic anhydride to protein. Further increase in ratio did not enhance the solubility index considerably; in fact, it leveled off when the ratio was above 0.5:1. The only exception is F-1-p in pH 5 solution, where the solubility index was continuously improved as a function of percent succinylation. It became 50% soluble at pH 5, which supposedly nears the isoelectric point of the native protein. In general, succinylation enhanced the solubility index more effectively in alkaline than in acidic condition. Succinylated F-1-p was almost completely soluble at pH 7.4 and higher, whereas F-2-p improved solubility to 70% under the same condition.

The effect of succinylation on the foaming properties of soluble tobacco leaf proteins appears to be statistically insignificant (Table II). However, it reduced foaming capacity slightly for both F-1-p and F-2-p, and yet the extent of modification exerted little effect. Foaming stability is consistently higher for the modified F-1-p than the native one, and an opposite relationship is apparent for F-2-p. Regardless of the extent of modification, F-1-p has better foaming properties than F-2-p. Progressive succinylation increased water absorption of leaf proteins (Table III). It occurred to a greater extent for F-2-p than for F-1-p. Native F-1-p had higher fat-binding capacity than the F-2-p counterpart, and succinylation doubled its capacity. This functionality was tripled for the acylated F-2-p derived from the 0.2:1 ratio treatment but showed a declining trend upon additional modification.

Table II. Effect of the Extent of Succinylation on the Foaming Capacity and Stability of Soluble Tobacco Leaf Proteins

leaf protein	ratio of succinic anhydride to protein, g/g	foaming cap.,[a] mL	foaming stability,[a] mL 10 min	30 min	1 h	2 h
F-1-p	0	92.7	43.2	40.6	36.3	29.1
	0.1	89.4	41.4	40.4	39.0	33.0
	0.2	89.2	43.2	40.0	37.8	34.4
	0.5	88.8	43.0	41.2	39.0	32.0
	1.0	88.4	41.6	39.6	37.6	34.0
	lsd 0.05	ns[b]	ns	ns	ns	ns
	lsd 0.01	ns	ns	ns	ns	ns
F-2-p	0	80.8	31.0	30.6	30.0	28.8
	0.1	73.6	27.0	25.4	25.0	24.2
	0.2	75.2	27.6	26.0	25.0	24.6
	0.5	75.7	27.6	25.6	25.0	24.6
	1.0	75.8	27.0	26.0	25.4	25.0
	lsd 0.05	ns	ns	ns	ns	ns
	lsd 0.01	ns	ns	ns	ns	ns

[a] Determined according to the method of Lawhon and Cater (1971). [b] Not significant.

Improvement of emulsifying capacity in proportion to the extent of succinylation is common for both leaf protein fractions (Table III). F-2-p increased in capacity by 49%, 79%, 93%, and 120% as the result of increment of succinylation. The corresponding values for F-1-p are 11%, 23%, 34%, and 56%. In almost all cases, the difference due to the stepwise increase of succinic anhydride in the treatments was statistically significant. In contrast, the emulsifying activity index of F-1-p was not significantly altered by acylation. Succinylated F-2-p, however, tripled the emulsifying activity index when it was modified at the 1:1 ratio.

DISCUSSION

Acylation of protein by succinate takes place readily for ϵ-amino of lysine and α-amino of polypeptides. Changing cationic amino groups to anionic carboxyl groups increases negative charges of polypeptides and leads to conformational alteration as well as subunit dissociation. The latter

Table III. Effect of the Extent of Succinylation on the Water Absorption, Fat-Binding Capacity, and Emulsifying Properties of Soluble Tobacco Leaf Proteins

leaf protein	ratio of succinic anhydride to protein, g/g	water absorpn,[a] %	fat-binding cap.,[b] %	emuls cap.,[c] mL of oil/g	emuls act. index,[d] m^2/g
F-1-p	0	327.9	167.4	480.9	127.4
	0.1	491.9	184.1	531.7	105.3
	0.2	433.9	232.2	589.2	118.4
	0.5	450.0	322.6	646.4	112.4
	1.0	570.1	356.9	749.2	108.6
	lsd 0.05	47.7	56.6	73.5	ns[e]
	lsd 0.01	69.4	82.4	107.0	ns
F-2-p	0	284.1	122.9	427.5	122.2
	0.1	514.8	299.0	635.9	174.7
	0.2	606.5	377.4	763.4	215.5
	0.5	621.9	316.0	824.2	289.9
	1.0	666.8	285.6	940.0	377.6
	lsd 0.05	65.1	30.4	52.9	51.0
	lsd 0.01	94.7	44.2	77.0	74.1

[a] Determined according to the method of Fleming et al. (1974). [b] Vantsinas and Nakai (1983). [c] Regenstein and Regenstein (1984). [d] Li-Chan et al. (1984). [e] Not significant.

phenomenon is evident for F-1-p as revealed by the nondissociating PAGE which substantiates the findings with succinylated soy glycinin (Franzen and Kinsella, 1976) and wheat gluten (Barber and Warthesen, 1982). On the SDS–PAGE gel, the dissociated large and small subunits of F-1-p reduced the mobility toward the anode as a function of the extent of succinylation (Figure 1). This reflects an increase of molecular weight due to succinate moieties, each of which can add 100 to the weight of the protein. Since the large subunit of tobacco F-1-p contains 25 lysines and 1 amino-terminal methionine (Amiri et al., 1984), a complete succinylation of these 26 amino acids amounts to an increase of 2600. This changes the weight of the large subunit from 56 000 to 58 600. On the basis of protein mobility, the increase in molecular weight is much greater. This requires acylation of nucleophilic groups of amino acids other than lysine and amino-terminal methionine. The hydroxyl groups of serine and threonine, the sulfhydryl group of cysteine, the phenol group of tyrosine, and the imidozole group of histidine are the potential candidates. This could add up to 8300 if all of them are succinylated. In other words, a maximal increase of the weight of the large subunit to 66 900 is possible. This is nearly a 20% increase for the 477 amino acid residues.

There are 123 amino acid residues for the small subunit of tobacco F-1-p with molecular weight of approximately 14 000 (Muller et al., 1983). Succinylation of lysine and amino-terminal methionine could increase the molecular weight by 1000. A complete succinylation of the remaining nucleophilic groups may have an additional increment of 2300. A molecular weight of 17 300 is more in line with the retarded mobility of the succinylated small subunit on the SDS–PAGE gel (Figure 1). The reduction in banding intensity of the succinylated small subunit is possibly attributable to the interference of acylation to the binding of Coomassie Brilliant Blue, a triphenylmethane dye. This is consistent with the underestimation of the quantity of succinylated F-1-p by use of the Bio-Rad protein assay method since both employ the same dye (Sheen, unpublished data). However, the banding intensity of the large subunit appears to be not affected by succinylation. Coomassie Brilliant Blue binds arginine-rich proteins more readily than lysine-rich ones (Reisner et al., 1975). The large subunit of tobacco F-1-p contains 30 residues of arginine, whereas there are only 5 arginines for the small subunit. Less arginine in combination with the possible steric hindrance due to the succinate moiety may explain the loss of banding intensity. Similarly, the effect of succinylation on dye binding coincided with the increase in molecular weight of F-2-p.

Molecular expansion and conformational change of F-1-p by succinylation are also affirmable by spectrophotometric shift and decrease in absorbance. In accordance with Freifelder (1982) these physical alterations indicate the shift of tyrosine and tryptophan into a more polar environment. Loss of molecular compactness owing to subunit dissociation by succinylation may cause the increase of bulk volume in F-1-p. The globular nature of F-1-p could limit the reassociation of hydrophobic residues among subunits; hence, the increase of bulk volume would be in proportion to the extent of succinylation. In contrast, at the high extent of chemical modification the decline in bulk volume of F-2-p may be attributed to the reassociation of hydrophobic residues among different globulins and albumins. This is in keeping with the decrease in fat-binding capacity (Table III).

The present study confirms the improvement in functionality of soluble leaf proteins by succinylation as reported by Franzen and Kinsella (1976b). Acylation of leaf protein results in electrostatic repulsion that facilitates the penetration of water molecules and enhances solubility. This is in agreement with the good foaming property and strong water absorption of modified F-1-p and F-2-p. Enhancement in water absorption improves emulsifying capacity, which substantiates a positive correlation between emulsifying capacity and the extent of succinylation in fish myofibrillar protein (Groninger, 1973). However, succinylation improves emulsifying activity index for F-2-p but showed no difference for F-1-p. The large and small subunits of F-1-p as globulins could limit molecular spread as "film" between polar and apolar interface and thus exert little influence on emulsifying activity index. On the other hand, the dissociation of hydrophobic residues among globulins and albumins of F-2-p at the interface of oil and water may be responsible for the progressive increase of emulsifying activity index in relation to the extent of succinylation.

The suitability of succinylated protein as food ingredient has been questioned with regard to nutritional deterioration. Siu and Thompson (1982) reported that whey protein concentrates succinylated in low and medium levels (15–37% ϵ-amino group) remain as good-quality protein with high net protein ratio. A high level of succinylation (74–83% ϵ-amino group) showed deleterious effects on protein quality. It is believed that acylases in mammalian tissue may not hydrolyze acylated ϵ-amino, α-amino, sulfhydryl, and hydroxyl groups to yield free amino acids. Therefore, long-term dose–response feeding studies have been suggested to assess the suitability of using succinylated protein in food systems. Barber and Warthesen (1982) reported that protein acylation with citraconic anhydride showed a substantial reversibility at pH 3. This would make citraconic anhydride more nutritionally acceptable as a modifying agent. In case of needs in the improvement of certain functionality of soluble leaf protein to meet food formulation, the modification of the protein with citraconic anhydride deserves investigation.

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