Preparation and Properties of Succinylated Fish Myofibrillar Protein

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Fish myofibrillar protein was reacted with succinic anhydride at 0° and a pH of 7.5–8.5 to form succinylated myofibrillar protein. This modified protein had the following dispersion properties: moderately rapid rehydration to form viscous aqueous dispersions that have a slightly opaque to water-like appearance; heat stability, as shown by the absence of coagulation or precipitation during heating at 100°; relatively good dispersion in the pH range of 6.0–8.5 (however, the presence of NaCl significantly lowered the viscosity); relatively high emulsification capacity as indicated by tests using a model system; and a relatively bland odor and flavor. The dried protein was organoleptically stable when stored at ambient temperature and with no special precautions to protect it from atmospheric oxygen and light. It was demonstrated that myofibrillar protein could be succinylated at various levels and that this degree of succinylation was related to functional property, such as emulsification capacity. The protein efficiency ratio for succinylated protein was somewhat lower than that of unsuccinylated fish protein.

The succinylation of protein is one approach to the controlled alteration of protein functionality. The modification of protein by N-acylation with succinic anhydride converts the positively charged amino groups to negatively charged residues (Means and Feeney, 1971). Since many of the functional characteristics of proteins depend upon the distribution and quantity of charged groups, changes in charges on these proteins result in modified proteins.

The early research and the main continuing effort in the succinylation of protein have been the use of succinylation as a tool in the study of the macromolecular nature of proteins. Habeeb *et al.* (1958) showed that succinylated proteins frequently exhibit increases in intrinsic viscosity and concomitant decreases in sedimentation coefficient at neutral or alkaline pH's resulting from a general unfolding and molecular expansion. Klotz and Keresztes-Nagy (1963) reported that succinylation of hemerythrin brings about dissociation into subunits. Extensive succinylation of bovine serum albumin was shown by Habeeb (1967) to increase its Stokes radius, increase the susceptibility of its disulfide bonds to reduction, and reduce its ability to precipitate antibovine serum albumin immunoglobulins.

The work of Oppenheimer *et al.* (1966, 1967) illustrates the effect of succinylation of a muscle protein. They showed that succinylation of chicken myosin resulted in a product that had increased viscosity, but a molecular size similar to that of unmodified myosin. In studies with actin, Mühlrad *et al.* (1968) demonstrated that a large molar excess of succinic anhydride was required to succinylate a significant percentage of the ϵ-amino groups of lysine.

The principal reaction of succinic anhydride with protein is through the ϵ-amino group of lysine (Grant-Greene and Friedberg, 1970). In various proteins, succinic anhydride has been shown to react with other functional groups. Gounaris and Perlmann (1967) showed that *O*-succinyltyrosines were formed during the succinylation of pepsinogen; however, these were observed to decompose spontaneously in 3–4 hr. They also showed that succinyl derivatives of aliphatic hydroxy amino acids of pepsinogen were formed during succinylation, but that these did not spontaneously hydrolyze. The reaction of succinic anhydride with the sulfhydryl groups of protein has been reported by Habeeb *et al.* (1967), Hass (1964), Meighen and Schachman (1970), and Mühlrad *et al.* (1968). However, in each of these investigations, relatively high anhydride–protein ratios were employed.

An example of the use of acylation to alter the functionality of a food product is the chemical modification of egg white with 3,3-dimethylglutaric anhydride (Gandhi *et al.*, 1968). It was demonstrated that the modified egg product had increased heat stability. In another example, Evans and Irons (1970) reported that N-succinylated egg yolk proteins were useful for the production of mayonnaises and salad dressings. Melnychyn and Stapley (1969) showed that acylated soybean protein has advantages as an ingredient in coffee whiteners because of improved flavor, odor, and dispersion characteristics. In a Carnation Co. patent (1970) there are listed a number of acylating agents, including succinic anhydride, that have been shown to be useful for preparing modified plant proteins which possess improved functional characteristics.

The purpose of this study was to prepare succinylated protein from fish myofibrillar protein and to examine some of the chemical and functional properties of these products. Emphasis was on the preparation of a stable, dry product that possesses desirable functional properties.

MATERIALS AND METHODS

Fish Muscle. Rockfish (*Sebastes* sp.) fillets of high quality were obtained from commercial sources. Fillets were either used immediately or were frozen rapidly and maintained at −15° until needed.

Preparation of Myofibrillar Protein. Fillets were comminuted by a single pass through a food grinder that was equipped with a plate having 0.15-cm orifices. Soluble protein was removed from the comminuted flesh in the following manner. The flesh was suspended (three times) in 5–10 vol of 0.1 *M* NaCl at 0° and the insoluble portion of the flesh was filtered out using cheesecloth. This partially washed material was suspended (two times) in about 3 vol of 0.1 *M* NaCl in a high-speed blender (equipped with a baffle to reduce entrapment of air) for 15–30 sec and the supernate was removed by centrifugation at 13,000 × *g*. This product, which contained mainly washed myofibrils, was suspended in 0.6 *M* NaCl at 0° and blended in a high-speed baffle-equipped blender for about 30 sec. Connective tissue and other nonsolubilized protein were removed by centrifugation at 13,000 × *g*. Typical preparations of this salt-solubilized myofibrillar protein contained 30–50 mg of protein/ml.

Succinylation. Salt-solubilized myofibrillar protein was reacted with solid succinic anhydride. During the 1–2-hr reaction period, the protein was stirred constantly, incremental amounts of anhydride were added during the first 30–90 min, the temperature was held near 0°, and the

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pH was maintained between 7.5–8.5 by the addition of 2 *N* NaOH. Ratios of 1:3–20 (anhydride–protein) were used. The termination of the reaction was indicated by a stabilization of the pH level.

Salt and Lipid Removal. The solubilized or dispersed succinylated protein was precipitated in the isoelectric range by adjustment of the pH to 4.4–4.5 using 0.1 *N* HCl and the supernate was removed by centrifugation at 13,000 × *g*. The precipitated succinylated protein was suspended (two times) in 5–10 vol of isopropyl alcohol in a blender and the suspension was stirred and heated in a water bath at 70–80° for approximately 15 min. The isopropyl alcohol was separated from the protein by filtration and the residual isopropyl alcohol was removed by successive washings (two to three times) with water.

Preparation of Sodium Salt. The sodium salt of the succinylated protein was prepared by the incremental addition of 2 *N* NaOH to an aqueous suspension of the protein while it was agitated in a high-speed blender. Typically, the final pH was adjusted to about 7.2–7.3. The sodium salt of the protein was normally dried by freeze drying. (It was also demonstrated that the sodium salt of the protein could be successfully drum dried.)

Analytical Determinations. Protein was determined by the method of Lowry *et al.* (1951). The degree of succinylation of protein was estimated by the method of Kakade and Liener (1969). Sulfhydryl groups were estimated by using Ellman's reagent in a method by Habeeb (1972). Ash was determined by heating the protein at 480° for 12 hr. Lipid was determined by extraction of the protein material for 12 hr using a solvent mixture of methanol–chloroform (2:1, v/v).

Emulsifying Capacity. A modification of the method of Webb *et al.* (1970) was used to determine emulsifying capacity. A 0.1–0.2-g sample of protein was dispersed in 200 ml of water in a 1-l. jacketed Waring Blendor cup which was equipped with electrodes. Blender speed was 16,000 rpm and the temperature 30° ± 2°. Oil was added at approximately 60 ml/min.

Viscosity Determinations. Viscosity measurements were made at 25° using a Brookfield Synchro-lectric Viscometer Model LVT. Protein material was dispersed in aqueous media by blending at low speed for 1–2 min. Entrapped air was removed from the dispersion by centrifugation at 500–5000 × *g*. All viscosities were determined at a spindle speed of 60 rpm, which is the highest speed for this instrument. Because of the thixotropic properties of these dispersions the viscosity data should be considered an "apparent viscosity."

Protein Efficiency Ratio. (Official Methods of Analysis, 1970). The succinylated protein was added to a nutritionally adequate basal diet at the 10% level.

RESULTS AND DISCUSSION

Succinylation Reaction Parameters. Crude succinylated myofibrillar protein was prepared as shown in Figure 1. The succinylated protein that is described in this work was prepared by succinylation of salt-solubilized myofibrillar protein. Limited efforts to succinylate myofibrils which were suspended in water gave reaction products that were not completely dispersible in aqueous media. Although it appeared that a technique might be developed to succinylate myofibrils, the idea was not pursued further at this time.

In general, under the conditions used in this work, the minimum weight of anhydride required to form a dispersible succinylated product was approximately 5% of the amount of protein in the reaction mixture. The content of the ε-amino group (lysine) in a reaction mixture has been used as a measure of the degree of succinylation. The relationship between the ratio of succinic anhydride to protein and the degree of succinylation is shown in Table I.

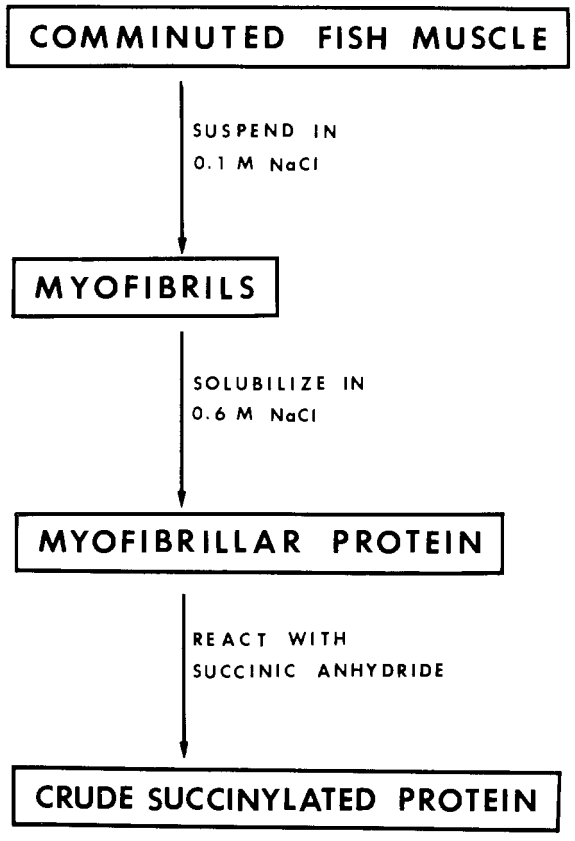

Figure 1. Preparation of myofibrils, myofibrillar protein, and crude succinylated protein.

Table I. Extent of Succinylation as a Function of the Succinic Anhydride–Protein Ratio

Anhydride–protein, w/w	ε-Amino groups reacted with anhydride, %
1:20	30
1:10	48
1:6.6	69
1:5	77

All of the succinylated protein described in this work was prepared at a reaction temperature of 0°. This temperature was selected to give maximum protection to the heat-labile myofibrillar protein. Habeeb *et al.* (1958) reported that the extent of the succinylation was virtually the same at 25° and 0°.

Removal of Excess Salt and Muscle Lipid from Succinylated Protein. By lowering the pH of the succinylated protein into the isoelectric range of pH 4.4–4.5, the protein was concentrated and the excess NaCl was removed from the protein (Figure 2).

Myofibrillar protein from fish muscle contains small-to-moderate amounts of lipid. Since this lipid is prone to rapid autoxidation, especially after the protein is dried, most of the lipid must be removed before a stable dry product can be achieved. Various solvents and conditions were tested for the removal of the lipid from the wet preparations of succinylated myofibrillar protein. Extraction of the isoelectric protein with hot isopropyl alcohol was shown to result in a product that was organoleptically stable during storage in the atmosphere at ambient temperature.

Formation of Sodium Salt of Succinylated Protein and Drying of the Salt Form. The adjustment of the pH of the succinylated protein from the isoelectric range to 7.2–7.3 converted the insoluble particles of succinylated protein, which were suspended in water, to a solubilized form (Figure 2). Since this solubilized form begins to take

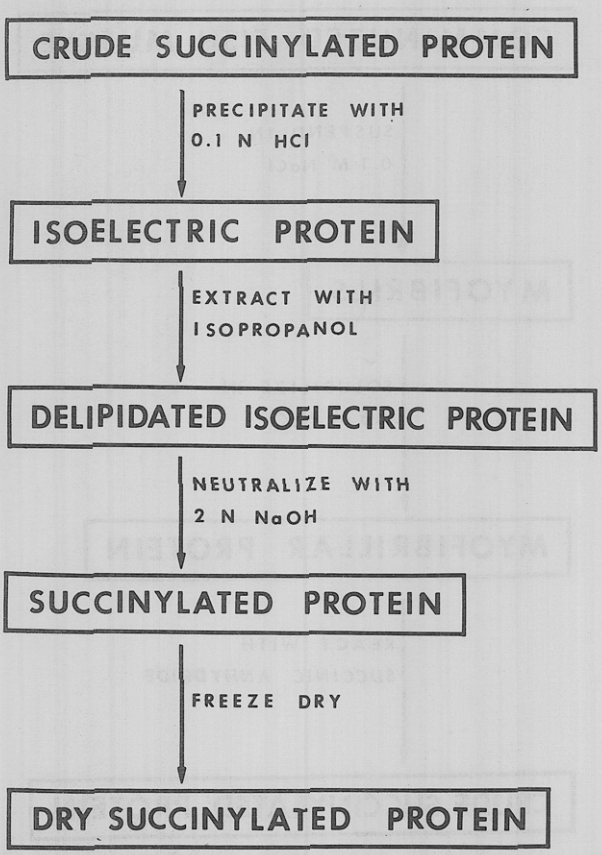

Figure 2. Delipidation and drying of succinylated protein.

Table II. Comparison of the Degree of Reaction of Succinic Anhydride with the ε-Amino Groups of Lysine and with Sulfhydryl Groups of Myofibrillar Protein

ε-Amino groups reacted, % of total	Sulfhydryl groups reacted, % of total
41	11
82	22
90	32

Table III. Mean Weight Gain, Food Consumed, and Protein Efficiency Ratio of Animals Fed Casein and Succinylated Protein[a]

	Casein	Succinylated protein
Avg daily weight gain	5.46 ± 0.016	3.72 ± 0.13
Avg daily food intake	14.63 ± 0.33	12.58 ± 0.39
PER	3.64 ± 0.06	2.86 ± 0.05
% of casein	100	79.0[b]

[a] 30–40% of the ε-amino groups of the protein in this sample were reacted with anhydride. [b] This value for fish is usually greater than 100%. For example, a value of 110% was obtained for red hake (Dubrow *et al.*, 1970).

on gel-like character and becomes viscous at a pH of about 5.5–6.0, sufficiently strong agitation must be used during the pH adjustment to assure homogeneous treatment.

Drying changes this succinylated protein gel into the form of a strong flexible highly porous mat. After disintegration of this mat in a high-speed blender, the succinylated protein is light and fluffy in appearance and has a high bulk density. A typical preparation has a composition of 85.3% protein, 11.2% ash, 1.5% lipid, and 2% moisture.

Reaction of Anhydride with Various Groups on the Protein. The major reaction of succinic anhydride is with the ε-amino group of lysine. Because of the limited information on the reaction of succinic anhydride with myofibrillar protein, some effort was made to determine the relative degree of reaction of the anhydride with lysine and with sulfhydryl groups. Under the conditions that the acylation reaction was carried out in this study, it was shown that succinic anhydride reacted to the extent of only one-third to one-fourth with sulfhydryl groups as compared to lysine groups (Table II).

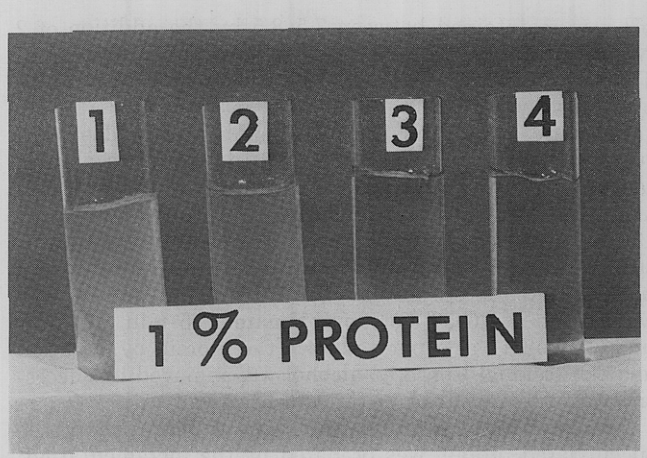

Figure 3. 1% dispersions of succinylated myofibrillar protein which were succinylated at the following levels of the total ε-amino groups of protein reacted with anhydride: no. 1, 30%; no. 2, 48%; no. 3, 69%; and no. 4, 77%.

Table IV. Apparent Viscosities of Protein Dispersions[a]

	% of succinylated protein in dispersion				
	0.2	0.5	1.0	1.5	2.0
Viscosity in centipoise at 25°	70	840	1700	4350	5500

[a] Approximately 50% of the ε-amino groups of the protein were reacted with anhydride.

Protein Efficiency Ratio. The nutritive properties of succinylated protein are an important consideration if this modified protein is to be used as a food ingredient. The protein efficiency ratio test results for a sample of succinylated myofibrillar protein, which had 30–40% of its ε-amino groups succinylated, are shown in Table III. These results indicated that some loss of protein quality occurred following succinylation, delipidation, and drying of the protein material.

There are two points that might be made relative to these data. First, since the amino acid balance is better in whole fish muscle than in myofibrillar protein, a somewhat lower PER might be expected for succinylated myofibrillar protein compared to whole muscle. Second, although no change in the nutritive properties of succinylated vegetable protein was reported (Carnation Co., 1970), this might be explained by the relative deficiency of lysine in many vegetable proteins.

Functional Properties of Aqueous Dispersion of Succinylated Protein. Dry succinylated myofibrillar protein is readily dispersible in water. Aqueous dispersions of 1–2% succinylated myofibrillar protein have a high viscosity and are slightly opaque to water-like in appearance (Figure 3). Heating to 100° does not cause coagulation or precipitation of the dispersed protein. Dispersions of the protein have a bland odor and flavor.

The relationship between the succinylated protein concentration and the viscosity is given in Table IV. The viscosity of the dispersions of succinylated protein did not vary greatly over the pH range of 6.0–8.5. As the pH is lowered from the pH of 6.0 to the isoelectric range of 4.4–4.5, there is a gradual decrease in the viscosity of the dispersion.

Table V. Effect of NaCl on the Apparent Viscosity of Succinylated Protein Suspension[a]

Protein concentration, %	Salt concentration, %	Viscosity, cP
1	0	1700
	0.1	600
	0.2	130
2	0	5500
	0.1	2000
	0.2	1150

[a] Approximately 50% of the ϵ-amino groups of the protein were reacted with anhydride.

Table VI. Emulsification Capacity[a] **of Succinylated Protein as a Function of the Degree of Succinylation**

ϵ-Amino groups reacted with anhydride, %	Emulsification capacity, g of oil/mg of protein
30	1.3
48	1.9
69	2.4
77	2.6

[a] Capacity for a soy protein isolate, using the same test system, was 0.35 g of oil/mg of protein.

The presence of sodium chloride in low concentrations greatly decreased the viscosity of dispersions of succinylated protein (Table V). Low levels of orthophosphate, polyphosphate, and nitrate also decreased viscosity.

The emulsification capacity of succinylated myofibrillar protein, as measured in a model system, is given in Table VI. These data indicated that the emulsification capacity was related to the degree of succinylation of the protein. Also, the emulsification capacity of the succinylated protein as measured in the model system was significantly greater than that for a soy protein isolate. Tsai *et al.* (1972) pointed out the difficulty in relating model system data, such as that reported here, to the protein functionality in a food product. Therefore these data only suggest that succinylated myofibrillar protein may have superior emulsification properties.

The emulsification capacity of succinylated protein was affected by pH and NaCl concentration; however, these effects appear to be only minor in magnitude. For example, at pH 4.0, the capacity was about 20% lower than that measured at neutrality. Concentrations of up to 3% NaCl decreased the capacity about 25%.

Potential Applications. These functional properties appear to make succinylated protein potentially useful as an ingredient in food and other systems. At the present time, more extensive investigations are being made on the food and other applications of succinylated protein.

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