Short Communication

Modification of Succinylated α_{s1} -Casein with Papain: Covalent Attachment of L-Norleucine Dodecyl Ester and Its Consequence

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The importance of modifying the functions of food proteins has been stressed in recent years. A possibility exists that even small changes in the chemical structures of proteins result in large changes in their functions.

The present work is an attempt to attach covalently a lipophilic group to a hydrophilic protein and thus maximize its surface-active function. The so-called "one-step process"^{1~4)} was applied for this purpose.

Succinylated α_{s1} -casein,^{5,6)} ca. 25,000 daltons, was used as a hydrophilic substrate; its homogeneity was confirmed by SDS-polyacrylamide gel electrophoresis.⁷⁾ L-Norleucine 1-¹³C-dodecyl ester, prepared from K¹³CN and 1-bromoundecane through four steps,^{8~10)} was used as a lipophilic nucleophile. CMR measurement of this preparation gave only one signal at a 65.2 ppm distance from TMS. Its PMR and IR spectra coincided exactly with those of authentic L-norleucine *n*-dodecyl ester.

The one-step process was carried out under the following conditions: medium, 20% acetone in 1 M carbonate (pH 9) containing 10 mM 2-mercaptoethanol; concentration of succinylated α_{s1} -casein, 20% (w/w); concentration of L-norleucine 1-¹³C-dodecyl ester, 0.25 M; concentration of papain (recrystallized, Sigma Chem. Co.), 0.02% (w/w); incubation temperature, 37°C; and incubation time, up to 30 min.

The 30 min-incubation mixture was lyophilized and subjected to Sephadex G-15 chromatography: column size, 4.0 × 50 cm (*V*₀ = 200 ml); solvent, 20% acetone in 0.1 M carbonate (pH 9); and temperature, 20°C. The *V*₀ fraction was collected which represented

FIG. 1. 100-MHz CMR Spectrum of a Polypeptide Fraction Containing the 20,000 Dalton Product.

The sample (200 mg) was dissolved in a 50 : 50 mixture (1 ml) of 0.01 M phosphate (pH 7.0) and D₂O used as an internal lock. A JEOL-FX-NMR spectrometer was used for the measurement. The spectrum shown is the result of 45,000 transients obtained by using 8K-frequency domain data points. Conditions: spectral width, 5000 Hz; recycle time, 0.69 sec; and flip angle, 38°.

about 95% of the incubation mixture on a dry-matter basis. It was confirmed that this high-molecular fraction was not contaminated by low-molecular species such as free L-norleucine 1-¹³C-dodecyl ester or free 1-¹³C-dodecanol. CMR measurement of this fraction gave a spectrum in which a distinct signal occurred at a 65 ppm distance from TMS, assignable to the 1-methylene ¹³C (Fig. 1). For further confirmation, a sufficient amount of unlabelled L-norleucine dodecyl ester or unlabelled 1-dodecanol was added to the high-molecular sample and the resulting mixture was subjected similarly to Sephadex chromatography, prior to the CMR measurement. Regardless of such an addition, a similar CMR spectrum resulted. No dilution effect was thus observed in this experiment. It is therefore concluded that L-norleucine 1-¹³C-dodecyl ester was covalently attached to a high-molecular product occurring in the 30 min-incubation mixture.

Analysis by SDS polyacrylamide gel electrophoresis⁷⁾ showed that this incubation mixture was comprised primarily of an approximately 20,000-dalton product as well as 25,000-dalton components. To investigate the 20,000-dalton product, the 30 min-incubation mixture was subjected to Sephadex G-75 chromatography: column size, 2.2 × 85 cm ($V_o = 95$ ml); solvent, 0.1 M carbonate (pH 9); and temperature, 20°C. After the 25,000-dalton components including intact succinylated α_{s1} -casein were eluted, the 20,000-dalton product followed with a $V_e/V_o = 1.34 \sim 1.36$. This product was analyzed for amino acid after hydrolysis with 6N HCl and for 1-dodecanol after hydrolysis with 1N NaOH. To quantify this alcohol gas chromatographic analysis was done as follows: apparatus, Shimadzu GC-4A; column, Silicone OV-17/3% Chromosorb-G packed in a glass tube (3 mm × 2 m); column temperature programming, 4°C/min elevation from 150°C; flow rate of carrier (N₂), 25.6 ml/min; retention time for 1-undecanol (internal standard), 5.1 min; and retention time for 1-dodecanol, 8.0 min. Also, the 20,000-dalton product was subjected to

ninhydrin treatment¹¹⁾ and, after hydrolysis with 1N NaOH, to treatment with carboxypeptidase A¹²⁾ in order to confirm that norleucine is actually liberated first. The results of these experiments are summarized in Table I.

An attempt was made to find out whether the 20,000-dalton product has an activity for emulsifying oil. This product (100 mg on a dry-matter basis) was homogenized with a 50:50 mixture (10 ml) of corn oil and 0.01 M phosphate (pH 7) as follows: apparatus, Nihon Seiki AM-5 blender; speed level, 10,000 rpm; revolution time, 3 min; and temperature, 25°C. The results, represented as cream-phase volume divided by total volume,¹³⁾ are shown in Table I.

TABLE I. PROPERTIES OF THE 20,000-DALTON POLYPEPTIDE, A MAJOR HIGH-MOLECULAR PRODUCT, FORMED FROM SUCCINYLATED α_{s1} -CASEIN BY TREATMENT WITH PAPAIN IN THE PRESENCE OF L-NORLEUCINE DODECYL ESTER

Items investigated	Results obtained
CMR spectrum	A clear 65-ppm signal observed ^a
Dodecanol content	0.88 mol/mol polypeptide
Norleucine content	0.85 mol/mol polypeptide
C-Terminal amino acid	Norleucine (in dodecyl ester form)
N-Terminal amino acid	Masked (succinylated) ^b
Emulsifying activity	0.63 ^c

^a cf. Fig. 1.

^b This polypeptide must bear the original succinylated arginine structure because of no ninhydrin response.

^c cf. Table II.

Further experiments were carried out with 5 min-, 15 min- and 30 min-incubation mixtures in order to correlate the contents of the 20,000-dalton product with the emulsifying activity. The content was roughly estimated from SDS-polyacrylamide gel electrophoresis followed by densitometry of stained zones, while the emulsifying activity was measured as described above. As the results shown in Table II indicate, a correlation apparently exists between the formation of the 20,000-dalton

TABLE II. FORMATION OF THE 20,000-DALTON PRODUCT AND INCREASE IN EMULSIFYING ACTIVITY DURING TREATMENT TO SUCCINYLATED α_{s1} -CASEIN WITH PAPAIN IN THE PRESENCE OF L-NORLEUCINE DODECYL ESTER

Incubation time (min)	Amount of the 20,000-dalton product formed ^a (%)	Emulsifying activity
0	0	0.39
5	9	0.42
15	33	0.50
30	49	0.59

^a Roughly estimated from SDS-polyacrylamide gel electrophoresis followed by densitometry of stained zones.

product and the increase in the emulsifying activity of incubation mixtures. It was noted that no increase in emulsifying activity results when succinylated α_{s1} -casein is incubated with papain in the absence of L-norleucine dodecyl ester.

All the data presented indicate that, under the intentionally abnormal conditions used in this study, papain can preferably catalyze the aminolysis of succinylated α_{s1} -casein by L-norleucine dodecyl ester, with formation of a surface-active 20,000-dalton product which is covalently attached to this lipophilic nucleophile. The amphiphilic function of the product is probably a result of the formation of a localized hydrophilic-lipophilic structure. The present study presents a good example that small changes in the chemical structures of proteins result in large changes in their functions.

Papers will be presented dealing with proteinaceous surfactants prepared from hydrophilic proteins by covalently attaching long-chain alkyl esters of hydrophobic amino acids through the one-step process.

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