Purification and Characterization of a Surface-active Macropeptide Produced from Succinylated α_{s1} -Casein by Modification with Papain

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Received April 13, 1982

The preceding paper described that when succinylated α_{s1} -casein, *ca.* 25,000 daltons, was modified with papain in the presence of L-leucine *n*-dodecyl ester (Leu-OC₁₂), an approximately 20,000-dalton macropeptide was formed as the main product. In the present work we have investigated its chemical structure and surface function. A treatment for purification at the petroleum ether/water interface gave an electrophoretically homogeneous 20,000-dalton macropeptide which functioned as a surfactant to emulsify corn oil as well as *n*-octane. Pulsed NMR and ESR studies demonstrated that the macropeptide, when used to emulsify *n*-octane in water, acted to restrict the mobility of those molecules involved in the emulsion. Various data from chemical analyses coupled with knowledge about the primary structure of α_{s1} -casein showed that the 20,000-dalton macropeptide was structured as succinyl-Arg¹-...-Phe¹⁴⁵-Leu-OC₁₂. A discussion is included to explain the surface function of this peptide in relation to its amphiphilic structure.

The preceding paper¹⁾ describes a modification treatment of succinylated α_{s1} -casein with papain under unusual conditions which produces an approximately 20,000-dalton macropeptide. The paper also mentions that when a similar enzymatic reaction is carried out in the presence of L-leucine *n*-dodecyl ester, which acts as an acyl-accepting nucleophile, the production of the 20,000-dalton macropeptide is enhanced. It was proposed that in this process the L-leucine *n*-dodecyl ester is covalently attached to the macropeptide and thus an amphiphilic structure results.

The present work is aimed at verifying this proposition. Discussions are made involving a relationship between the amphiphilic structure of the 20,000-dalton macropeptide and its surface activity.

In this paper, succinylated α_{s1} -casein and L-leucine *n*-dodecyl ester are abbreviated as suc- α_{s1} -casein and Leu-OC₁₂, respectively.

MATERIALS AND METHODS

Substrate. Bovine α_{s1} -casein (variant B) was prepared from fresh defatted milk according to the method of Zittle and Custer.²⁾ This preparation was immediately succinylated with succinic anhydride³⁾ which amounted to 10 times the quantity of the protein amino groups on a molar basis. The resulting degree of succinylation was 98.0% when measured by a method using trinitrobenzenesulfonic acid.⁴⁾ Dialysis in running water followed by lyophilization afforded a suc- α_{s1} -casein preparation which was electrophoretically homogeneous.

Nucleophile. Leu-OC₁₂ was synthesized by the C-activation method using *p*-toluenesulfonic acid.⁵⁾ The product obtained by this method was crystallized from acetone-water at 5°C, prior to being used as a nucleophilic reagent in the following enzymatic reaction.

Enzymatic reaction. A papain preparation (Sigma Chemical Co.) with a BAEE unit of 22.0 at pH 6.2 and 25°C was used as the enzyme. The reaction was carried out for up to 30 min in the presence of Leu-OC₁₂ under conditions simulating those adopted in the preceding study.¹⁾ A similar reaction was conducted in the absence of Leu-OC₁₂ for 10 min to obtain a protein hydrolysate.

Electrophoresis. SDS-15% polyacrylamide gel electro-

phoresis was carried out to follow this enzymatic reaction as well as to verify the homogeneity of the substrate and the 20,000-dalton product. The procedures used were essentially similar to those reported by Weber and Osborn.⁶ Coomassie brilliant blue R-250 (Nakarai Chemicals) was used as the staining reagent.

Purification of the 20,000-dalton product. The enzymatic reaction was carried out for 5 min and the resulting product was used as the sample for purification at the petroleum ether/water interface. The sample (2.5 g on a dry-matter basis) was dispersed in 50 ml of 0.01 M phosphate (pH 7.0) and the dispersion poured to 50 ml of petroleum ether. The mixture was emulsified with a blender (Nihon Seiki AM-5) at 10,000 rpm for 3 min at room temperature. Immediately, the emulsion was centrifuged at $8000 \times g$ for 5 min at room temperature to obtain a floating cream layer. Evaporation of the petroleum ether from this cream gave a partially purified 20,000-dalton macropeptide. The same purification method was repeated four more times.

Pulsed NMR measurement. The purified 20,000-dalton macropeptide (500 mg) was dispersed in 2.5 ml of 0.01 M phosphate (pH 7.0) and emulsified with 2.5 ml of *n*-octane by ultrasonication under the following conditions: apparatus, Tokyo Riko Ultrasonicator Type 50-6; output frequency, 10 kHz; anode voltage, 1000 V; anode current, 225 mA; bath temperature, 10°C; and sonication time, 10 min. The resulting emulsion was measured for an average spin-lattice relaxation time (T_1) of the water protons involved. A Plaxis model PR-1005 was used for the measurement and operated at 10.72 MHz and 10°C in the standard 90° - τ - 90° pulse sequence, with a τ value varying from 0.01 to 0.3 sec. The signal amplitude corresponding to the water protons at each τ value was corrected by subtracting a half signal amplitude of *n*-octane used alone from that of the emulsion.⁷

ESR measurement. 2-(10-Carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxy methyl ester (Syva) was used as an oil-soluble spin probe. This was dissolved in *n*-octane at a concentration of 0.4 mM. A dispersion of the 20,000-dalton macropeptide (500 mg) in the above phosphate buffer (2.5 ml) was mixed with the *n*-octane (2.5 ml) containing the spin probe and the mixture then ultrasonicated as described. The emulsion formed was immediately submitted to measurement with an ESR spectrometer (JEOL JES-PE-3X). Every measurement was conducted at 10°C and the spectra were recorded in the 9.2~9.3 GHz range using a modulation frequency of 100 kHz and incident microwave power of 10 mV. Rotatory correlation times (τ_c) were worked out by analyzing the involved spectral parameters according to the method of Waggoner *et al.*⁸

Emulsifying activity measurement with corn oil. In the course of the enzymatic reaction, samplings were made at

appropriate intervals and each sample was immediately lyophilized. The lyophilized sample (125 mg) was dispersed in 2.5 ml of 0.01 M phosphate (pH 7.0) and then emulsified with corn oil (Showa Sangyo Co.) at 40°C. A blender (Nihon Seiki AM-5) was used for the emulsification and operated at 10,000 rpm for 5 min. The result was presented in terms of the emulsifying activity index proposed by Pearce and Kinsella.⁹

Partition in an octane/water system. A lyophilized sample (50 mg) was dispersed in the above phosphate buffer (2.5 ml) and the dispersion mixed with *n*-octane (2.5 ml). The mixture was emulsified with a blender (Nihon Seiki AM-5) at 10,000 rpm for 3 min and the emulsion centrifuged at $8000 \times g$ for 15 min; all these operations were made at room temperature. From the bottom of the centrifuge tube, a 1 ml portion of the water layer was carefully sampled by aspiration and the sample quickly lyophilized. This lyophilized sample was dissolved in water (10 ml) and the solution measured for optical density at 280 nm.

N-Terminal amino acid analysis. A product resulting from the 5 min enzymatic reaction was dialyzed in running water for 2 days and lyophilized. The lyophilized sample (300 mg) was dissolved in 0.4 M NaHCO₃ (3 ml). To the solution was added ethanol (6 ml) containing 1-fluoro-2,4-dinitrobenzene (0.1 mg). The mixture was vigorously shaken under the conditions proposed by Sanger.¹⁰ The resulting product, after lyophilization, was hydrolyzed with 6 N HCl at 105°C for 16 hr. The hydrolysate obtained was divided into an ethyl acetate-soluble fraction and a water-soluble fraction by a conventional method.¹¹ Both fractions were independently analyzed for DNP-amino acids with silica-gel thin-layer plates (Merck Art. 11844); the ethyl acetate-soluble fraction was developed with chloroform-methanol-acetic acid (95:5:1) and the water-soluble fraction with 1.5 M phosphate (pH 6).

C-Terminal sequence analysis. The 20,000-dalton macropeptide was saponified with a sufficient amount of 0.5 N NaOH at 37°C for 3 hr to remove the dodecyl moiety. In a control experiment, however, the same sample was used without saponification. Both the saponified and the unsaponified macropeptide were treated with a carboxypeptidase-A preparation (Sigma Chemical Co.), which had been pretreated with DFP and recrystallized. The conditions used were very similar to those instructed by Fraenkel-Conrat *et al.*¹²

Determination of amino acid composition. The 20,000-dalton macropeptide (1 mg) was dissolved in 6 N HCl (1 ml) in a test tube. The solution was degassed by a freeze-thaw technique, prior to hydrolysis at 110°C for 24 hr, and the hydrolysate analyzed with an amino acid analyzer (Hitachi KLA-5A).

RESULTS

Limited degradation of substrate

When suc- α_{s1} -casein, ca. 25,000 daltons, was incubated with papain in the presence of Leu-OC₁₂, it appeared that a limited degradation

FIG. 1. Time-course Formation of a 20,000-Dalton Product from Succinylated α_{s1} -Casein, ca. 25,000 Daltons, by Treatment with Papain.

FIG. 2. Co-chromatography on Silica Gel of an Ethyl Acetate-soluble DNP-Amino Acid Fraction as a Test Sample (T) and an Authentic Di-DNP-Tyr Sample (A). Detection was made by spraying a 5% solution of fluorescein in ethanol and taking a picture in the dark.

reaction occurred within 10 min of incubation, leading to formation of an approximately 20,000-dalton product (Fig. 1).

The N-terminal amino acid analysis showed that, while suc- α_{s1} -casein gave no significant amount of α -DNP-amino acid, the 5 min reaction mixture afforded a large amount of an ethyl acetate-soluble DNP-amino acid fraction. The main DNP-amino acid constituting this fraction was found to be di-DNP-Tyr (Fig. 2). It was confirmed that the water-soluble DNP-amino acid fraction gave no particular zones of α -DNP amino acids. Thus, it was concluded that the substrate, suc- α_{s1} -casein, underwent degradation at a peptide bond, involving the amino group of a certain tyrosine residue.

Increase in emulsifying activity

Samplings were made at intervals during the enzymatic reaction in the presence of Leu-OC₁₂. Each sample was measured for its activity to emulsify corn oil, with the result that the emulsifying activity index increased smoothly from 39 m²/g in the sample before incubation to 59 m²/g in the sample after incubation for 30 min (Table I). No increase in this index resulted when the substrate, suc- α_{s1} -casein, was incubated with papain in a similar system which, however, lacked the nucleophile, Leu-OC₁₂.

TABLE I. CHANGES IN CORN OIL-EMULSIFYING ACTIVITY OBSERVED DURING THE INCUBATION OF SUCCINYLATED α_{s1} -CASEIN WITH PAPAIN IN THE PRESENCE OR ABSENCE OF LEUCINE DODECYL ESTER (Leu-OC₁₂)

Incubation time (min)	Emulsifying activity index (m ² /g)	
	In the presence of Leu-OC ₁₂	In the absence of Leu-OC ₁₂
0*	39	39
5	47	40
10	53	40
30	59	40

* Original succinylated α_{s1} -casein.

TABLE II. PARTITION IN AN OCTANE-WATER SYSTEM

Sample	Partition (%)	
	In cream layer	In water layer
Succinylated α_{s1} -casein	56	44
20,000-Dalton macropeptide	73	27
Hydrolysate*	48	52

* Resulting from the 10 min papain-treatment of succinylated α_{s1} -casein in the absence of Leu-OC₁₂.

Instead of corn oil, *n*-octane was used as the phase to be dispersed. To emulsify the *n*-octane, the following three samples were used: suc- α_{s1} -casein (before the enzymatic reaction); the macropeptide (I₅) mentioned below; and the hydrolysate produced from suc- α_{s1} -casein by treatment with papain in the absence of Leu-OC₁₂. The result was that these samples were partitioned in aqueous and cream phases with the ratios shown in Table II. This indicates that the second sample was more surface-active than the others and more adsorbable on the octane/water interface.

Interfacial purification of the 20,000-dalton macropeptide

The 5 min reaction product was selected as a source of the surface-active 20,000-dalton macropeptide. Repeated concentration at the interface of petroleum ether and water resulted in a gradually purified 20,000-dalton macropeptide (Fig. 3). The final product (I₅) that resulted from five repetitions was electrophoretically homogeneous (Fig. 3) and considered to be appropriate as a sample for the following examinations.

Emulsifying function of the 20,000-dalton macropeptide

The 20,000-dalton macropeptide purified above was evaluated first for its function of emulsifying corn oil. The result was that the macropeptide itself had an emulsifying activity index as high as 62 m²/g (*cf.* Table I).

The 20,000-dalton macropeptide also func-

FIG. 3. Electrophoretic Patterns Showing the Degree of Purification of the 20,000-Dalton Macropeptide.

S, succinylated α_{s1} -casein before the enzymatic reaction; P, the whole mixture after the enzymatic reaction; I₁, the product after interfacial purification; I₂ and I₅, products after the purification was repeated twice and five times, respectively.

TABLE III. SPIN-LATTICE RELAXATION TIMES (T_1) FOR WATER AND ROTATIONAL CORRELATION TIMES (τ_c) FOR THE SPIN PROBE OBSERVED BEFORE AND AFTER EMULSIFICATION OF AN OCTANE-WATER DISPERSION WITH THE 20,000-DALTON MACROPEPTIDE*

Parameter	Before emulsification	After emulsification
T_1 (sec)	0.21	0.05
τ_c (sec)	5.7×10^{-11}	0.9×10^{-9}

* Used at a concentration of 10% in the water phase.

tioned as an effective emulsifier when *n*-octane was used. The molecular interaction involved in the emulsification was verified by two independent methods of instrumental analysis. Firstly, a pulsed NMR analysis of the water protons in an octane-macropeptide-water system showed an average T_1 value of 0.21 sec before emulsification and a much smaller value (0.05 sec) after emulsification (Table III). Secondly, an ESR analysis of the spin probe dissolved in the same system gave a τ_c value of 5.7×10^{-11} sec before emulsification and a much larger value (0.9×10^{-9} sec) after emulsification (Table III). These results

FIG. 4. Time-course Liberation of Amino Acids from the 20,000-Dalton Macropeptide by Treatment with Carboxypeptidase A.

indicate that the mobilities of the water and *n*-octane molecules are restricted in some measure due to emulsification with the 20,000-dalton macropeptide.

Chemical structure of the 20,000-dalton macropeptide

The purified 20,000-dalton macropeptide (I_5 in Fig. 3) was analyzed for its chemical structure. It was found that carboxypeptidase A failed to act on this macropeptide. Saponified with NaOH, it underwent the action of carboxypeptidase A, with the liberation of leucine, phenylalanine and tyrosine in that order (Fig. 4). No free amino acids were liberated when the macropeptide was treated with leucine aminopeptidase (Sigma Chemical Co.) under normal conditions.¹³⁾

Table IV shows the amino acid composition found for the 20,000-dalton macropeptide; the composition calculated for $H \cdot Arg^1 \cdots Phe^{145} \cdot Leu \cdot OH$ is shown for reference.

DISCUSSION

Bovine α_{s1} -casein (variant B) is a single polypeptide with a known sequence of 199 amino acid residues.¹⁴⁾ The molecular weight of suc- α_{s1} -casein is calculated as 25,120 daltons for the case when all its amino groups are

TABLE IV. AMINO ACID COMPOSITION FOUND FOR THE 20,000-DALTON MACROPEPTIDE AND THAT CALCULATED FOR THE PEPTIDE FRAGMENT, $H \cdot Arg^1 \cdots Phe^{145} \cdot Leu \cdot OH$

Amino acid	Found (mol/mol)	Calculated
Asx	10.1	10
Thr	1.3	1
Ser	10.2	11
Glx	35.0	33
Pro	9.7	10
Gly	6.0	6
Ala	5.5	6
Val	9.8	10
Met	3.3	4
Ile	8.5	9
Leu	13.7	14
Tyr	3.3	4
Phe	4.5	5
Trp	0	0
Lys	12.6	13
His	4.3	5
Arg	4.9	5

succinylated. This value may apply to the suc- α_{s1} -casein we used, since its degree of succinylation was 98%.

Modification of the suc- α_{s1} -casein with papain in the presence of $Leu \cdot OC_{12}$ can give rise to a 20,000-dalton product which, because of its surface activity, can be concentrated by repeated purification at the petroleum ether/water interface (Fig. 3).

The chemical structure of the 20,000-dalton macropeptide is characterized in summary by: (1) having no free amino group at the N-terminal; (2) being masked at the C-terminal carboxyl group, probably in the form of a dodecyl ester¹⁾; and (3) having a C-terminal sequence of $-Tyr-Phe-Leu$. It is clear from the preceding paper¹⁾ that this terminal leucine residue originates in the nucleophile, $Leu \cdot OC_{12}$. The rest can be found in α_{s1} -casein as its partial sequence, $Tyr^{144}-Phe^{145}$. The amino acid composition found for the 20,000-dalton macropeptide was almost identical with that calculated for an $Arg^1 \cdots Phe^{145}$ moiety attaching one molecule of leucine (Table IV).

All these data indicate that in the initial

FIG. 5. Covalent Incorporation of Leucine Dodecyl Ester into a Succinylated α_{s1} -Casein Molecule and the Resulting Formation of a 20,000-Dalton Macropeptide with an Amphiphilic Structure.

The positions of ionizable amino acid residues on α_{s1} -casein are marked with vertical bars.

process of the papain-catalyzed reaction, suc- α_{s1} -casein undergoes degradation at the peptide bond involving Phe¹⁴⁵ and Tyr¹⁴⁶, with the probable formation of a macropeptidyl-papain intermediate. It follows that an approximately 5000-dalton peptide leaves, whose N-terminal amino acid is Tyr¹⁴⁶. In fact, the N-terminal analysis of the 5 min reaction mixture showed that di-DNP-Tyr occurred as the main α -DNP-amino acid (Fig. 2). It is also estimated that the intermediate is attacked by Leu-OC₁₂ when it is present and converted into the 20,000-dalton product with a leucine dodecyl moiety attached at the C-terminal.

The schematic diagram in Fig. 5 illustrates the primary mode of this enzymatic reaction leading to formation of the 20,000-dalton macropeptide as the main product and the 5000-dalton leaving peptide. In the diagram, the ionizable amino acid residues are marked with vertical lines. Apparently, the macropeptide, suc-Arg¹...-Phe¹⁴⁵ is richer in ionizable amino acid residues and, therefore, more hydrophilic than suc- α_{s1} -casein itself. It is probable that the covalent attachment of a highly hydrophobic leucine dodecyl moiety to this hydrophile results in the formation of such an amphiphilic structure as illustrated (Fig. 5).

α_{s1} -Casein per se bears an amphiphilic structure. This structure is maintained even after

succinylation and may probably cause the surface activity of suc- α_{s1} -casein. In fact, suc- α_{s1} -casein was found to show a certain degree of emulsifying activity (Table I). The 20,000-dalton macropeptide, however, had a much greater emulsifying activity than suc- α_{s1} -casein itself. This is ascribable to the formation of a proper amphiphilic structure, with a highly hydrophobic dodecyl chain attached to a hydrophilic macropeptide (Fig. 5). Thus, the papain-catalyzed modification of suc- α_{s1} -casein with incorporation of a hydrophobic group is effective in enhancing the surface activity of this protein through alteration of a part of its chemical structure.

The modification of gelatin in this way offers a similar example; it is possible to implant a distinct surface activity to the hydrophilic protein, gelatin, by covalent attachment of a hydrophobic leucine *n*-alkyl ester.¹⁵⁻¹⁸⁾ The resulting product can be used as a non-lipid, proteinaceous surfactant to prepare a variety of emulsion foods.^{16,17)}

The data obtained in the present work will add knowledge basic to an understanding of the function of proteinaceous surfactants produced from food proteins by enzymatic modification.

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