Proteolysis in Structural Analysis of α_{s1}-Casein Adsorbed onto Oil Surfaces of Emulsions and Improvement of the Emulsifying Properties of Protein

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α_{s1}-Casein has a unique amphiphilic structure which may provide excellent emulsifying properties. Structural analysis of α_{s1}-casein adsorbed to emulsified oil globules and improvement of the emulsifying properties of α_{s1}-casein were undertaken by using proteolysis. Enzymic cleavage of α_{s1}-casein adsorbed to oil globules of the emulsion was compared with that dissolved in an aqueous solution by using trypsin and chymotrypsin. Thirteen peptide bonds of the adsorbed α_{s1}-casein in the emulsion were hardly cleaved and were probably among definite regions inaccessible to the proteases. Based on the results, a preliminary model is proposed for the structure of α_{s1}-casein at an oil/water interface. Emulsifying activity (EA) of α_{s1}-casein was increased by pepsin digestion. A peptide fraction (PF) separated from the peptic hydrolysate, being composed of α_{s1}-CN(fl-23) and a small amount of other peptides, showed an EA similar to that of α_{s1}-casein at neutral pH. Removal of the small amount of the peptides from PF resulted in a marked decrease of EA. However, the addition of the removed peptides to α_{s1}-CN(fl-23) restored the EA. Some synergistic effect between α_{s1}-CN(fl-23) and the other peptides in emulsification was suggested.

Proteases could be widely used to distinguish the external moieties from the internal moieties of a polypeptide in lipid-protein complexes. We applied this method to elucidate the structure of proteins adsorbed at oil globule surfaces by emulsification. α_{s1}-Casein, the major casein in bovine milk, is known to have good emulsifying properties (1). Since the primary structure has already been established (2) and a unique amphiphilicity of the structure has been pointed out (three hydrophobic regions are discernible, roughly including residues 1–44, 90–113 and 132–199), the emulsion system composed of α_{s1}-casein and lipids is a good model for studying the structure of proteins adsorbed at the oil/water interface of an emulsion. Here we review our recent studies on the utility of proteases for elucidating the structure of α_{s1}-casein at an oil/water interface and propose a detailed model of this structure. We also summarize our results on the improvement of emulsifying properties of α_{s1}-casein by proteolysis and the emulsifying peptides derived from α_{s1}-casein by protease action.

APPLICATION OF PROTEASES TO ANALYSES OF STRUCTURE OF α_{s1}-CASEIN AT AN OIL/WATER INTERFACE (3)

The susceptibility of the peptide bonds in α_{s1}-casein to proteases was compared between two systems. One was a solution system where α_{s1}-casein was present in an aqueous medium as a soluble form, and the other was an emulsion system where α_{s1}-casein was adsorbed to the emulsified oil globules. The emulsion was prepared by homogenizing 16 ml of 1% α_{s1}-casein at pH 7.0 with 4 g of soybean oil by using a Polytron PT-20 (Kinematica GmbH) at full speed (19500 rpm) for 8 min at 30 C. Figure 1 shows the high performance liquid chromatography (HPLC) patterns of tryptic peptides from α_{s1}-casein in the solution system (a) and in the emulsion system (b). Peptides were fractionated and purified according to the HPLC patterns. These peptides were identified by comparing their amino acid composition and C-terminal sequence with those of the peptides which were expected to be derived from α_{s1}-casein in accordance with the substrate specificity of trypsin. The same experiment was carried out by using chymotrypsin (data not shown).

The HPLC patterns of the digests in the emulsion system were different from those in the solution system for both trypsin and chymotrypsin digestion. The peptide bonds split by trypsin or chymotrypsin were

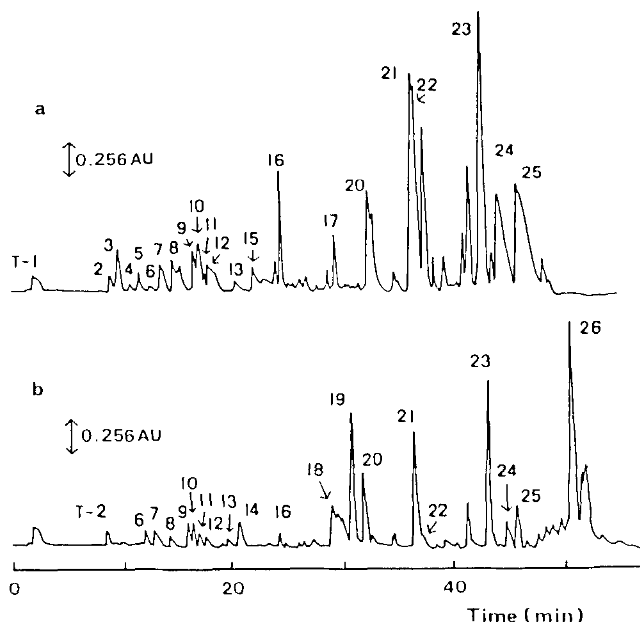

FIG. 1. HPLC patterns of tryptic peptides from α_{s1}-casein in the solution system (a) and the emulsion system (b) (3). Mobile phase: 0.1% trifluoroacetic acid/95% acetonitrile; slope: 0.75% acetonitrile/min; ultraviolet absorbance at 230 nm. The tryptic peptides were identified by amino acid residue numbers as follows: peak T-1, 103; T-2, 1-3; T-3, 104-105; T-4, 101-102; T-5, 103-105; T-6, 101-103; T-7, 80-83; T-8, 4-7; T-9, 125-132; T-10, 84-90; T-11, 37-42; T-12, 120-124; T-13, 35-42; T-14, 80-90; T-15, 59-79; T-16, 43-58; T-17, 106-119; T-18, 43-79; T-19, 194-199; T-20, 104-119; T-21, 194-199; T-22, 8-22; T-23, 152-193; T-24, 91-100; T-25, 133-151; and T-26, 133-193.

determined according to the results of identification of peptide fragments produced by the action of the above proteases. Their positions in the primary structure of α_{s1}-casein are shown in Figure 2. Forty-seven peptide bonds were cleaved by either trypsin and chymotrypsin in the solution system, but cleavage of 13 of these was difficult in the emulsion system (shown by arrowheads in Fig. 2).

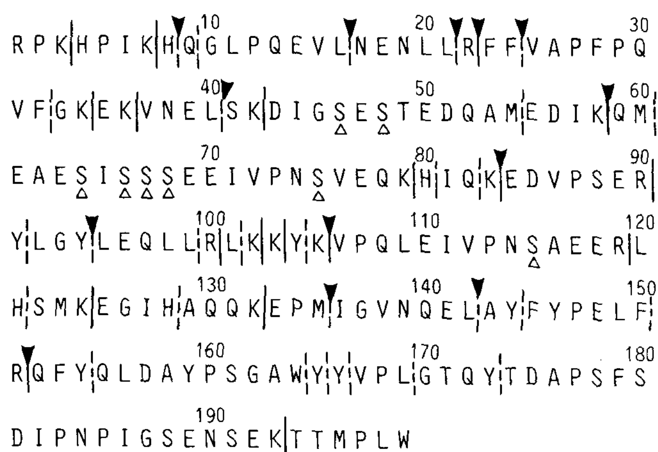

FIG. 2. Primary structure of bovine α_{s1}-casein B (3). Solid lines indicate the positions cleaved by trypsin in the solution system. Dashed lines indicate the positions cleaved by chymotrypsin in the solution system. Arrowheads indicate the positions cleaved in the solution system, but difficult for cleavage in the emulsion system. △, phosphoserine residue.

Based on the above results, the polypeptide chain of α_{s1}-casein adsorbed to an oil/water interface is schematically represented in Figure 3a. Peptide bonds cleaved in both systems are placed on the side of the aqueous phase and those for which cleavage was difficult in the emulsion system are placed in contact with the oil phase.

The validity of the model structure in Figure 3a will now be discussed from the viewpoint of hydrophobicity of the peptide portion. The profile of average hydrophobicity (4) of α_{s1}-casein is shown in Figure 3b. Hydrophobic amino acids were localized near residues 21–25, 94, 135 and 142. Since the peptide linkages around these hydrophobic residues were not susceptible to proteases in the emulsion system, they must be located in the sites for adsorption to oil. On the other hand, residues 164 to 166, which consisted of two tyrosines and one tryptophan, were cleaved in the emulsion system. This suggests that these residues did not associate with the oil phase in spite of the high hydrophobicity of their neighborhood. Evaluating the hydrophobicity of peptide portions does not always seem to reveal the adsorption sites of proteins. In spite of such difficulties, we can tentatively identify some adsorption sites of α_{s1}-casein at an oil/water interface. It may be concluded that proteolytic digestion is a valuable means for studying protein structure in emulsion systems.

APPLICATION OF PROTEASES TO THE IMPROVEMENT OF EMULSIFYING ACTIVITY OF α_{s1}-CASEIN (5,6)

Limited cleavage of α_{s1}-casein was carried out by using pepsin, chymosin or plasmin. An emulsion was pre-

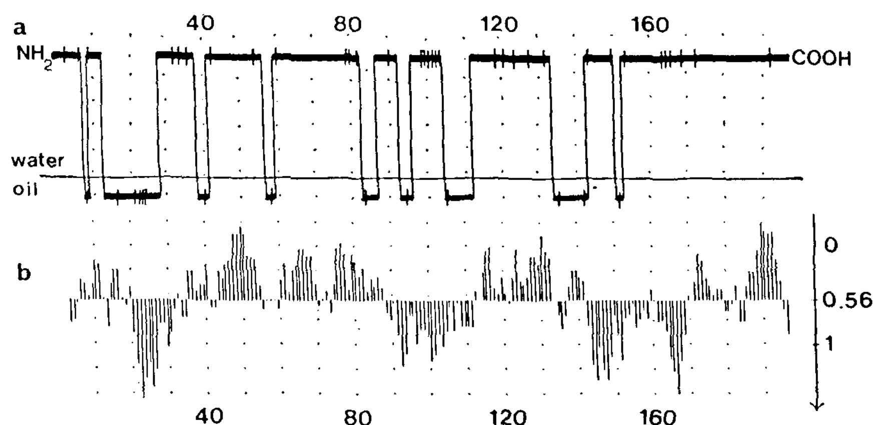

FIG. 3. Schematic representation of an α_{s1}-casein chain adsorbed to an oil/water interface (a) and a hydrophobic profile of α_{s1}-casein (b) (3). The vertical bars on the side of water in (a) indicate the positions of peptide bonds cleaved by proteases in both the emulsion and solution systems. The vertical bars on the side of oil in (a) indicate the positions cleaved in the solution system, but difficult for cleavage in the emulsion system. Hydrophobicity value was calculated by the constant determined by Rekker (4). The bar length indicates the hydrophobicity (downward) or hydrophilicity (upward) of peptide regions. The values are the averages for six residues, and are plotted in the middle of the six residues from which they were derived.

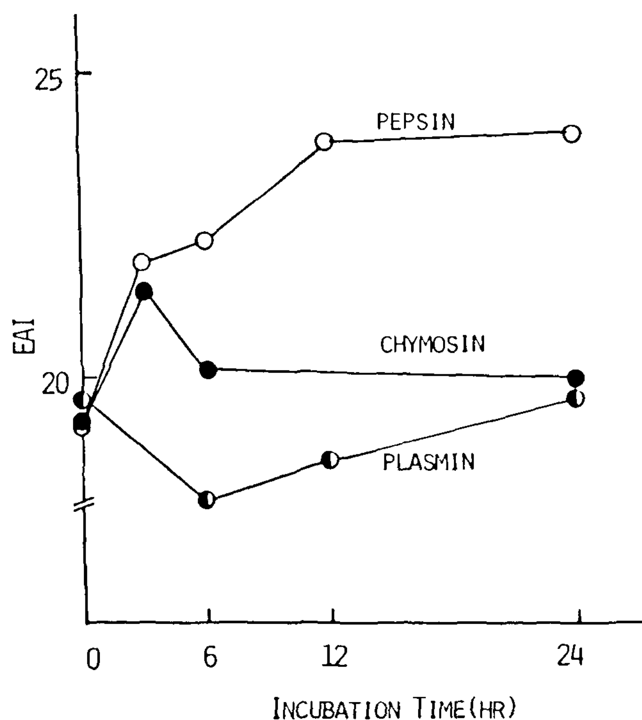

FIG. 4. Effect of protease digestion on the EAI α_{s1}-casein. Each point is the average of three observations (5). Pepsin and chymosin digestion were carried out in 0.1 M sodium acetate buffer, pH 6.4, at 30 C. Plasmin digestion was in 5mM phosphate buffer (pH 7.0) at 37 C. Emulsifying activity was evaluated according to the procedure described by Pearce and Kinsella (7) with a slight modification.

pared by homogenizing a 2% (w/w) peptide solution and 20% (w/w) soybean oil at 30 C with a Polytron PTA-7 for 3 min at full speed.

No marked increase in the emulsifying activity index (EAI) was observed by plasmin or chymosin digestion of the α_{s1}-casein (Fig. 4). However, the EAI was increased by pepsin hydrolysis. It was assumed that some peptides having good emulsifying activity were produced in the pepsin digests, and isolation of the peptides was attempted.

The supernatant of the pepsin hydrolysate of α_{s1}-casein at pH 4.6 contained residues 1-23 of α_{s1}-casein [α_{s1}-CN(f1-23)] as a major peptide. However, reverse-phase HPLC suggested that small amounts of other peptides were also present. This fraction, designated as crude α_{s1}-CN(f1-23), was subjected to a DEAE-Sephacel column. The chromatographic pattern is shown in Figure 5, and HPLC patterns of the resulting fractions are shown in Figure 6. α_{s1}-CN(f1-23) was eluted in Fraction I as a pure peptide. Fraction II contained other materials with retention times differing from that of α_{s1}-CN(f1-23). Amino acid analysis of the major component of Fraction II suggested that the component was α_{s1}-CN(f154-199), the C-terminal fragment of α_{s1}-casein. Fraction III was not identified.

The emulsifying activities of the crude and purified α_{s1}-CN(f1-23) were compared. As shown in Table 1, the EAI of the crude α_{s1}-CN(f1-23) fraction was decreased

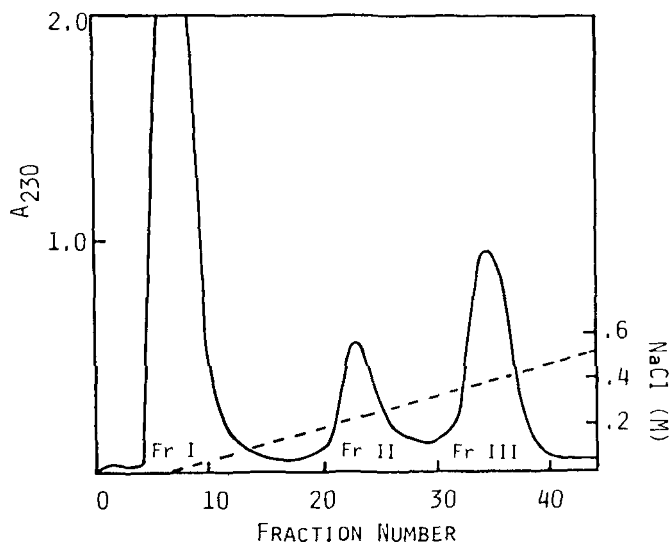

FIG. 5. DEAE-Sephacel chromatographic pattern of crude α_{s1}-CN(f1-23) fraction (6). Flow rate: 20 ml/hr; 6 ml/tube. The peptides were eluted with a linear gradient of NaCl.

by removing the coexistent materials, suggesting that α_{s1}-CN(f1-23) itself has very poor emulsifying properties. However, when Fraction II [whose major component was α_{s1}-CN(f154-199)] was added to purified α_{s1}-CN(f1-23), the EAI was increased to the level of the

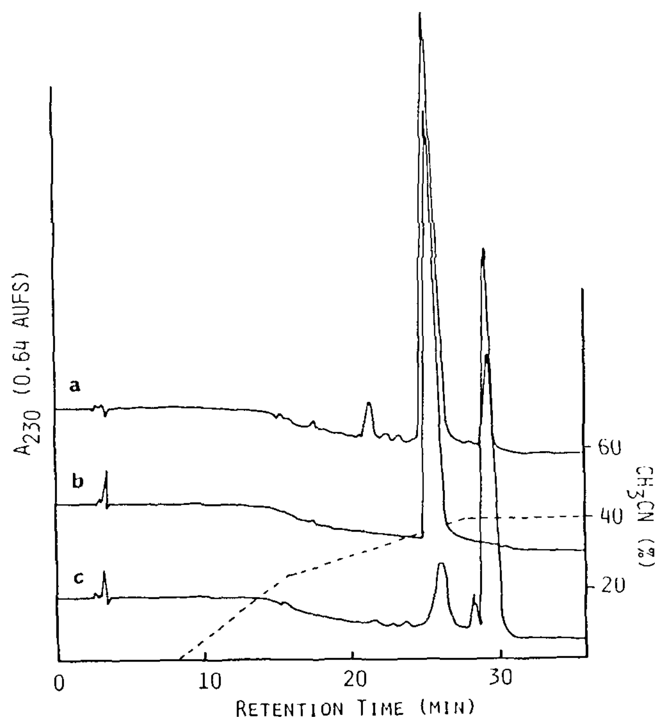

FIG. 6. HPLC patterns of the DEAE-Sephacel chromatography fractions (6): (a), crude α_{s1}-CN(f1-23) fraction; (b), Fraction I (purified α_{s1}-CN(f1-23); (c), Fraction II. The gradient profile (acetonitrile concentration) is shown by the dotted line.

TABLE 1

Emulsifying Activity of α_{s1}-CN(f1-23) in the Presence or Absence of Other Peptides (6)

	Emulsifying activity index (EAI)[a] (m^2/g)
Crude α_{s1}-CN(f1-23) fraction	23.19 ± 0.72
Fraction I [purified α_{s1}-CN(f1-23)]	5.41 ± 0.76
Fraction I + Fraction II[b]	16.51
Glycomacropeptide (as control)	15.86 ± 0.42
Fraction I + glycomacropeptide[c]	23.41 ± 0.89

[a]Measured at a total peptide concentration of 2% (w/w). Values are the average of three preparations, except that the value for Fraction I + Fraction II is the average of two preparations.

[b]Fraction I:fraction II = 3:1 (w/w)

[c]Fraction I:glycomacropeptide = 1:1 (w/w)

crude fraction. This suggests that the high EAI value of crude α_{s1}-CN(f1-23) was due to some synergistic effect between α_{s1}-CN(f1-23) and other peptides such as α_{s1}-CN(f154-199). When the glycomacropeptide, κ-CN(f106-169), was added to α_{s1}-CN(f1-23), the EAI value of α_{s1}-CN(f1-23) was also increased. These results suggest that the emulsifying properties of the peptide, α_{s1}-CN(f1-23), are strongly influenced by the coexistence of certain other peptides.

Since peptides have smaller molecular sizes and simpler structure than proteins, we had thought that peptides showed simpler behavior in emulsification than proteins. The present study, however, demonstrated that the peptides of only 23 residues also had very complex emulsifying properties, being affected by coexistent peptides and other factors. In order to utilize peptides as functional ingredients for various food products, more knowledge of the peptide functionality is essential.

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