Changes in the molecular weight of guayule rubber can be minimized by avoiding prolonged postharvest storage without refrigeration, avoiding light and use of antioxidants. Of course, all steps in the determination of rubber content must be standardized, including the age and type of material to be harvested.

Another feature of the viscometric method is the omission of an acetone extraction prior to dissolution of rubber in cyclohexane. Acetone extraction was to remove resins and other substances which affect the rubber determinations in methods such as gravimetric and IR techniques. Only the presence of dissolved rubber affected the viscosity.

Acetone solubles, either redissolved alone in cyclohexane or together with rubber, did not immediately affect the viscosity of the solution. They did, however, accelerate the time dependent degradation of rubber solutions, as measured by a loss of viscosity. Keller and Stephens (1982) state that the unsaturated fatty acids in guayule resin are primarily responsible for the accelerated degradation of rubber by oxidizing to form peroxide compounds which function as oxidation initiators. Acetone extraction of plant material did not prevent the degradation. Conversely it also acted to accelerate the loss of viscosity. Therefore, its inclusion in the extraction of rubber was further unwarranted.

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Relationship between Surface Functional Properties and Flexibility of Proteins Detected by the Protease Susceptibility

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The relationships between the surface properties and the flexibility of proteins were investigated. An attempt to detect the flexibility of protein structure was made by the protease digestion method. Ovalbumin and lysozyme were not susceptible to protease, suggesting rigid or folded molecules, while κ-casein, β-lactoglobulin, and bovine serum albumin were susceptible to protease, suggesting flexible molecules. The digestion velocity of proteins by α-chymotrypsin and trypsin was closely correlated. Good correlations were observed between the foaming power and emulsifying activity and the digestion velocity of proteins. These results suggest that the flexibility of protein structure detected by protease digestion may be an important structural factor governing the foam formation and emulsification.

INTRODUCTION

A number of studies have been done on the relationship between protein structural and functional properties such as emulsification and foam formation (Keshavarz and Nakai, 1979; Kato and Nakai, 1980; Nakai, 1983; Townsend and Nakai, 1983; Shimizu et al., 1983). Summarizing the information obtained from these studies, the most important structural factor of functional properties seems to be the protein hydrophobicity. It is reasonable to assume that the surface hydrophobicity of proteins plays a governing role triggering emulsification and foaming (Kato and Nakai, 1980; Nakai, 1983; Kato et al., 1983a). Amphiphilic proteins possessing high surface hydrophobicity are forcefully adsorbed at the interface between oil or air and water and cause a pronounced reduction of interfacial or surface tension that readily facilitates emulsification and foaming (Kato et al., 1983a; Shimizu et al., 1983). However, the functional properties of proteins can not be ac-

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counted for by the surface hydrophobicity alone. For example, α-lactalbumin shows good emulsifying and foaming properties, although it has low surface hydrophobicity. Therefore, structural factors other than surface hydrophobicity should also be considered to establish relationships between the structural and functional properties of proteins.

It is well-known that proteins are susceptible to denaturation at oil–water and air–water interface. If proteins undergo surface denaturation, the surface hydrophobicity increases and results in good emulsifying and foaming properties. Thus, the susceptibility of surface denaturation at the oil–water and air–water interface may be involved in the functional properties of proteins. It can be presumed that flexible protein molecules are susceptible to denaturation at the interface while the rigid protein molecules are not. However, since methods to estimate the flexibility of proteins have not been established, the relationship between the functional properties and flexibility of proteins has not been satisfactorily investigated, although the importance of flexibility has been pointed out (Shimizu et al., 1981; Townsend and Nakai, 1983). Since flexible proteins are more susceptible to proteases than rigid proteins, the protease susceptibility method seems to be promising in the detection of protein flexibility.

This paper describes the relationship between the functional properties (emulsifying and foaming properties) and flexibility of proteins determined by the protease digestion method.

MATERIALS AND METHODS

Ovalbumin was prepared from fresh egg white by the crystallization method in sodium sulfate and recrystallized five times (Kekwick and Cannan, 1936). Lysozyme was prepared from fresh egg white by a direct crystallization method and recrystallized five times (Alderton and Fevold, 1946). Ovotransferrin was prepared from fresh egg white by the method of Azari and Baugh (1967). 11S globulin was prepared from soybean by the method of Thanh et al.(1975). κ-Casein was prepared from fresh milk by the method of Zittle and Custer (1963). Serum albumin(bovine) and α-lactalbumin were purchased from Sigma Chemical Co. (St. Louis, MO). β-Lactoglobulin (bovine) was from Miles Laboratories (United Kingdom). α-Chymotrypsin (1240 chymotrypsin NF units/mg) was also from Miles laboratories. Trypsin (12300 BAEE units/mg) was from Sigma Chemical Co.

The acetylation of proteins was carried out by the method of Riordan and Vallee (1972). Two hundred milligrams of protein was dissolved in 10 mL of 0.5 g of saturated sodium acetate solution. Protein solution was cooled in an ice bath and treated with 200 mg of acetic anhydride in limited amounts over a 10-min interval with stirring at 0 °C for 1 h. The pH of the reaction solution was kept near 8.0. The product was dialyzed against distilled water and lyophilized.

Protease digestion was performed as follows: 4 mL of 0.1% protein solution in 0.05 M Tris-HCl buffer, pH 8.0, was added 250 μL of 0.1% α-chymotrypsin or trypsin solution. Enzymatic reaction was carried out at 38 °C for a given time in the protein–enzyme ratio of 16:1. After protease digestion, 4 mL of 4% aqueous trichloroacetic acid was added to remove the native protein and then the precipitates were removed by filtration with filter paper (Toyo Roshi Ltd, No. 5b). The amount of peptides and amino acids in the filtrate was estimated by the Lowry method. The extent of digestion was indicated as the digestion percentages of total protein. The 100% digestion was determined by digesting for 1 h the proteins heating at 90 °C for 10 min. The digestion velocity of proteins was linearly increased with the time assayed within 5 min. Therefore, the digestion velocity was represented as the digestion percents per 1 min, calculating from the initial slopes of digeston curves against time.

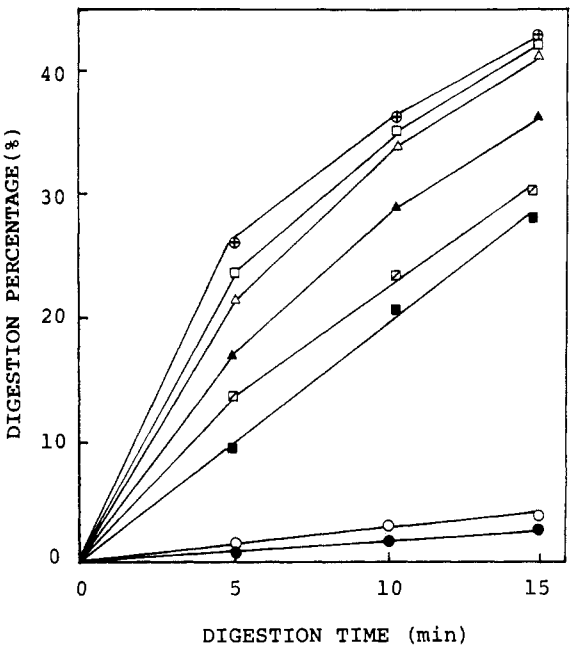

Figure 1. Time course of proteolytic digestion with α-chymotrypsin of various proteins: (○) ovalbumin; (●) lysozyme; (■) ovotransferrin; (▨) 11S globulin; (▲) α-lactalbumin; (△) bovine serum albumin; (□) β-lactoglobulin; (⊕) κ-casein.

The foaming power of proteins was determined by measuring the electric conductivity of foams when air was introduced into 5 mL of a 0.1% protein solution in $^1/_{15}$ M phosphate buffer, pH 7.4, in a glass filter (G-4) at a constant flow rate, 90 cm^3/min, for 15 s (Kato et al., 1983b). Foaming power was indicated as the conductivity of foams produced immediately after air was introduced into protein solution for 15 s.

The emulsifying activity of proteins was determined by the method of Pearce and Kinsella (1978). To prepare emulsions, 1.3 mL of corn oil and 4 mL of 0.1% protein solution in $^1/_{15}$ M phosphate buffer, pH 7.4, were homogenized in an Ultra Turrax (Hansen & Co., West Germany) at 12000 rpm for 1 min at 20 °C. One tenth milliliter of emulsions was taken from the bottom of the container immediately after homogenization and diluted with 0.1% SDS solution. The turbidity of diluted emulsions was then measured at 500 nm. The emulsifying activity of proteins was indicated as the absorbance at 500 nm of diluted emulsions.

RESULTS AND DISCUSSION

The time course of α-chymotrypsin digestion of various proteins is shown in Figure 1. There were big differences in the susceptibility to protease between various proteins. Ovalbumin and lysozyme were not susceptible to α-chymotrypsin, while κ-casein, β-lactoglobulin, and bovine serum albumin were greatly susceptible to α-chymotrypsin (Figure 1). The digestion velocity of proteins by α-chymotrypsin and trypsin was compared (Table I). A good correlation was obtained between the digestion velocity of proteins by α-chymotrypsin and trypsin. The correlation coefficient is 0.95 and significant ($p < 0.01$). This suggests that the protease susceptibility of native proteins is mostly dependent on the conformation rather than the primary amino acid sequence of substrates. Since protein unfolding is known to increase protease susceptibility (Privalov,

Table I. Digestion Velocity of Proteins by α-Chymotryposin and Trypsin

proteins	digestion velocity, %/min	
	α-chymotrypsin	trypsin
ovalbumin	0.5	0.4
lysozyme	0.3	0.1
ovotransferrin	1.7	1.3
11S globulin	2.3	2.8
α-lactalbumin	2.9	3.0
serum albumin(bovine)	4.0	3.2
β-lactoglobulin	4.3	3.5
κ-casein	4.8	3.4

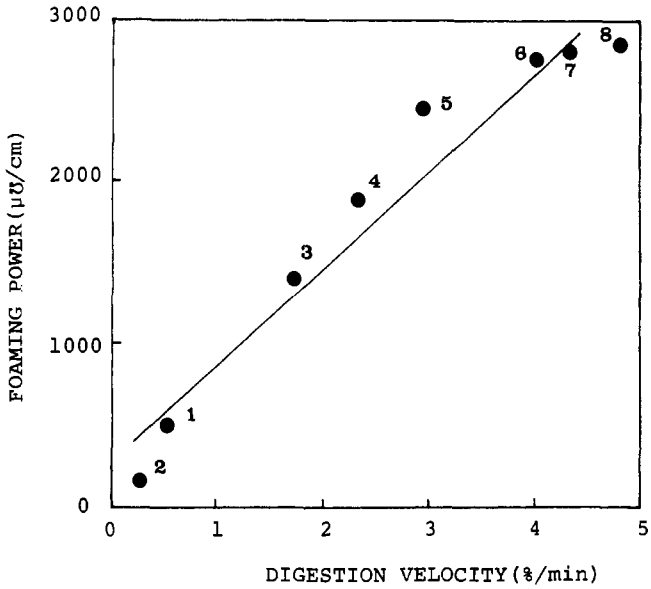

Figure 2. Relationship between foaming power and digestion velocity of various proteins: 1, ovalbumin; 2, lysozyme; 3, ovotransferrin; 4, 11S globulin; 5, α-lactalbumin; 6, bovine serum albumin; 7, β-lactoglobulin; 8, κ-casein.

1979), it is probable that ovalbumin and lysozyme have a somewhat tighter or more folded structure and thus become fewer proteolytically susceptible conformational substrates. Imoto et al. (1976) proposed that native and denatured protein molecules coexist even under physiological conditions and that the native denatured conformation (N ⇌ D) transition under mild conditions can be detected by protease digestion. The amounts of the protein in the D state reflecting the shift in the N ⇌ D transition to the right are too small to detect by routine optical methods. Since protease digestion proceeds only by the all-or-none type mechanism and protease digests only the unfolded molecules (Imoto et al., 1976), the amounts of N ⇌ D transition can be detected by protease digestion method. Therefore, the susceptibility to protease may reflect the flexibility of protein structure, because the extent of the N ⇌ D transition reflects the flexibility of protein molecules. Actually, it has been reported that β-lactoglobulin, bovine serum albumin, and α-lactalbumin are flexible (Whitney, 1977; Bloomfield, 1966; Takase et al., 1976; Takesada et al., 1973). β-Lactoglobulin is presumed to be susceptible to denaturation above neutral pH (Whitney, 1977). Bovine serum albumin is also presumed to be flexible according to low angle X-ray diffraction (Bloomfield, 1966). Although the chemical structure of α-lactalbumin is very similar to that of lysozyme, the stability of α-lactalbumin is low (Takase et al., 1976) and it exchanges all the internal hydrogens much faster than lysozyme (Takesada et al., 1973), suggesting the flexibility of structure. Ueno and Harrington (1984) also have reported that proteolytic enzyme can probe the structural

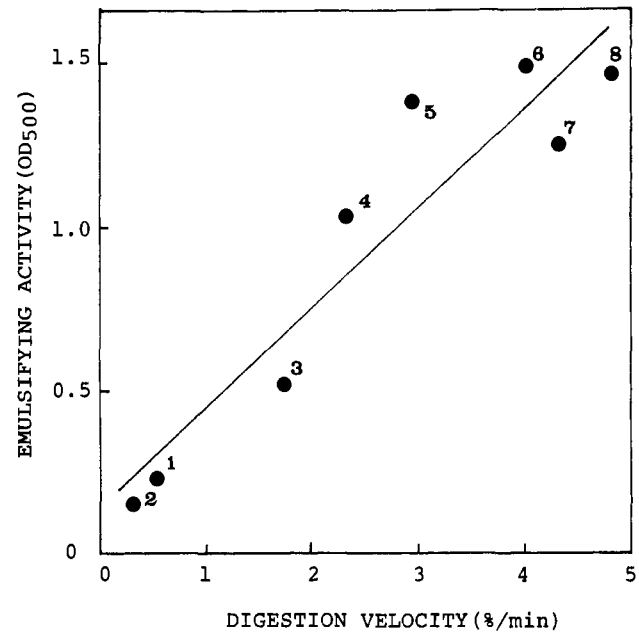

Figure 3. Relationship between emulsifying activity and digestion velocity of various proteins: 1–8, same as Figure 2.

Table II. Effect of Acetylation on Digestion Velocity and Ellipticity of Proteins

proteins	digestion velocity,[a] %/min	ellipticity[b] (222 nm)
ovalbumin		
native	0.5	100
acetylated	1.9	95
lysozyme		
native	0.3	100
acetylated	3.0	97
ovotransferrin		
native	1.7	100
acetylated	2.7	98
11S globulin		
native	2.3	100
acetylated	3.9	80

[a] Determined by α-chymotrypsin. [b] Indicated as percentages of ellipticity at 222 nm to each native protein.

changes arising from the flexibility in myosin rod and that the enzyme-probe method is of value in detecting a slight structural changes that would be difficult or impossible to monitor by other physical methods. Thus, the flexibility of proteins may be detected by protease digestion.

Good correlations were observed between the foaming power and emulsifying activity and the digestion velocity of proteins determined by α-chymotrypsin (Figures 2 and 3). The correlation coefficient is 0.96 ($p < 0.01$) for the foaming power and 0.93 ($p < 0.01$) for the emulsifying activity. These results suggest that the flexibility of protein structure detected by protease digestion is an important governing factor of the foam formation and emulsification. It is probable that the flexible protein molecules are susceptible to denaturation at the oil–water or air–water interface while the rigid protein molecules are not. Since the surface hydrophobicity of protein increases with denaturation at the interface, the emulsifying and foaming properties may be improved. Therefore, the importance of the flexibility is consistent with that of surface hydrophobicity as structural factors of the functional properties of proteins.

The relationships between the functional properties and digestion velocity of proteins were further investigated by using acetylated proteins. As shown in Table II, the digestion velocity of proteins was greatly increased by ace-

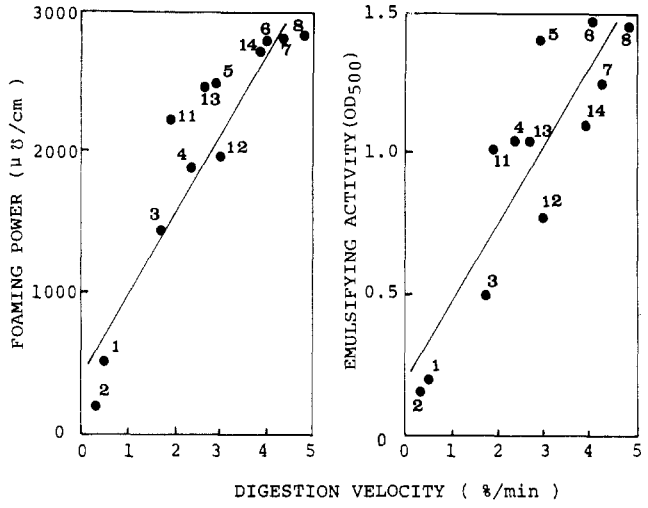

Figure 4. Relationship between foaming power or emulsifying activity and digestion velocity of proteins combined with four acetylated proteins: 1–8, same as Figure 2; 11, acetylated ovalbumin; 12, acetylated lysozyme; 13, acetylated ovotransferrin; 14, acetylated 11S globulin.

tylation, while changes in the ellipticity at 222 nm, reflecting the helix content, were very small. This result suggests that protease digestion is sensitive to slight structural changes which can not be detected by routine optical methods. Imoto et al. (1976) reported that acetylated lysozyme showed full lysozyme activity, suggesting no conformational changes, although the digestion velocity increased about 10 times. They concluded from this result that alteration of the net charges of lysozyme by acetylation caused a shift of the N $\rightleftharpoons$ D transition to the right. This may be the case for ovalbumin, ovotransferrin, and 11S globulin. The flexibility of protein molecules may increase with acetylation, although the gross conformational changes are not observed. This is the reason why the digestion velocity of proteins increases greatly by acetylation. Figure 4 shows the relationships between the digestion velocity and functional properties of proteins, the combined four acetylated proteins. Good correlations were obtained between the foaming power, emulsifying activity, and the digestion velocity of proteins. The correlation coefficient is 0.91 ($p < 0.01$) for the foaming power and 0.87 ($p < 0.01$) for the emulsifying activity. Thus, it was further confirmed that the flexibility of the protein structure detected by protease digestion was closely correlated with the foaming and emulsifying properties. Taking into account the correlation coefficient in Figures 2, 3, and 4, the foaming properties seem to correlate with the flexibility of proteins more closely than the emulsifying properties.

We have proposed that the surface hydrophobicity of proteins plays a governing role on the foam formation and emulsification (Kato and Nakai, 1980; Kato et al., 1983a). As shown in this paper, the flexibility of protein structure should also be added to a factor governing the foam formation and emulsification. The surface hydrophobicity may be a static factor and the flexibility may be a dynamic factor in the surface properties of proteins. Since flexible proteins are endowed with high surface hydrophobicity by a mild effector, e.g., an air–water or oil–water interface, the surface hydrophobicity is ultimately an important factor to obtain good foaming and emulsifying properties. Interestingly, the high hydrophobic proteins, such as bovine serum albumin and β-lactoglobulin, were susceptible to protease. The hydrophobic proteins seem to cause an unstable conformation which increases the flexibility of proteins due to the hydrophobic regions at the surface of the protein molecules.

Registry No. Lysozyme, 9001-63-2; protease, 9001-92-7; α-chymotrypsin, 9004-07-3; trypsin, 9002-07-7.

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