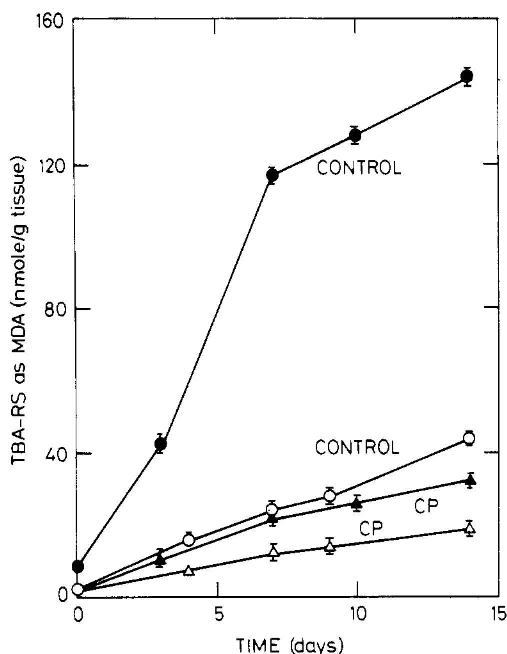

Figure 3. Effect of ceruloplasmin (150 U/g) on in situ lipid peroxidation in minced turkey muscle tissues: ●, control, minced muscle (run 1); ▲, minced muscle (run 1) in the presence of ceruloplasmin; ○, control, minced muscle (run 2); △, minced muscle (run 2) in the presence of ceruloplasmin.

Reactions 5 and 6 resemble the activity of a hydroperoxidase like glutathione peroxidase, which decomposes hydroperoxides to nonradical compounds. The decomposition of hydroperoxides by heme proteins in the presence of ascorbic acid has been documented (O'Brien, 1969).

In situ minced turkey muscle lipid peroxidation was inhibited by ceruloplasmin (Figure 3). This result was obtained with two different sources of turkey muscles, one of which tends to oxidize rapidly, the other more stable. In both samples, ceruloplasmin prevents between 76% and 56% of the lipid peroxidation process. As a great part of the oxidation is prevented by low concentration of EDTA (Kanner et al., 1988) and ceruloplasmin, we conclude that "free" iron ions are the major prooxidant factor in minced turkey muscles. Our results open a new way for the utilization of ceruloplasmin, a natural byproduct of animal blood, as a natural antioxidant for muscle foods.

Registry No. Fe, 7439-89-6; ceruloplasmin, 9031-37-2; ascorbate, 50-81-7.

LITERATURE CITED

Apgar, M. E.; Hultin, O. H. *Cryobiology* **1982**, *19*, 154.
Barber, A. A. *Arch. Biochem. Biophys.* **1961**, *96*, 38.
Bidlack, W. R.; Okia, R. I.; Hochstein, B. *Biochem. Biophys, Res. Commun.* **1973**, *53*, 459.
Buege, J. A.; Aust, S. D. *Methods Enzymol* **1978**, *52*, 302.
Cannistraro, S.; Ianzini, F.; Indovina, P. L. *Stud. Biophys.* **1981**, *86*, 163.
Gutteridge, J. M. C. *Chem. Biol. Interact.* **1985a**, *56*, 113.
Gutteridge, J. M. C. In *Handbook of Methods for Oxygen Radical Research*; Greenwald, R. A., Ed.; CRC: Boca Raton, FL, 1985b; p 303.
Hazan, B. M.Sc. Thesis, Hebrew University of Jerusalem, Israel, 1986.
Kanner, J.; Harel, S. *Arch. Biochem. Biophys.* **1985**, *237*, 314.
Kanner, J.; Harel, S.; Hazan, B. *J. Agric. Food Chem.* **1986**, *34*, 506.
Kanner, J.; Shegalovich, I.; Harel, S.; Hazan, B. *J. Agric. Food Chem.* **1988**, companion paper in this issue.
Marceau, N.; Aspin, N. *Biochem. Biophys. Acta* **1973a**, *293*, 338.
Marceau, N.; Aspin, N. *Biochem. Biophys. Acta* **1973b**, *328*, 351.
Nakano, H.; Ogita, K.; Gutteridge, J. M. C.; Nakano, M. *FEBS Lett.* **1984**, *166*, 232.
O'Brien, P. J. *Can. J. Biochem.* **1969**, *47*, 485.
Osaki, S.; Johnson, D. A.; Frieden, E. *J. Biol. Chem.* **1966**, *241*, 2745.
Vidlakova, M.; Erazimova, J.; Horki, J.; Placer, Z. *Clin. Chim. Acta* **1972**, *36*, 61.
Witte, V. C.; Krause, G. F.; Bailey, M. E. *J. Food Sci.* **1970**, *35*, 582.
Yamashoji, S.; Kajimoto, G. *FEBS Lett.* **1983**, *152*, 168.

Received for review March 9, 1987. Revised manuscript received November 30, 1987. Accepted January 21, 1988. Contribution from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel, No. 1954-E, 1987 series. This work was supported by the U.S.–Israel Binational Agricultural Research and Development Fund (BARD), Grant No. I-801-84.

Limited Proteolysis of Ovalbumin by Pepsin

Naofumi Kitabatake,* Kaoru Indo, and Etsushiro Doi

The hydrolysis of hen egg white ovalbumin by porcine pepsin was examined at various pHs. Changes in molecular size of the ovalbumin occurring during hydrolysis were investigated by SDS–polyacrylamide gel electrophoresis. A strictly limited hydrolysis was observed at pH 4. Only a single peptide bond in the original ovalbumin (MW 45 000) was cleft, and a peptide with a molecular weight of about 3000 was released. Both the released peptide and the residual protein (MW 42 000) were resistant to further hydrolysis by prolonged incubation or addition of more pepsin. The cleavage site was between His-22 and Ala-23 of the ovalbumin amino acid sequence.

Enzymatic modification is a useful method to improve and upgrade the functional and nutritional properties of food protein; in particular, proteolytic hydrolysis is widely applied for such modification (Yamamoto, 1975; Kay, 1982; Adler-Nissen, 1986). Kitabatake and Doi (1985) prepared a transparent gel from pepsin-treated ovalbumin and egg white by heating although nonproteolyzed proteins gave turbid gels. However, it is known that an extensive hydrolysis of protein gives deteriorative effects: formation of a bitter peptide or loss of a functional property. Proteolytic activities are required to be controlled in some cases. For this purpose limited proteolysis is available.

The transparent gel obtained from pepsin-treated ovalbumin, described above, gave no bitterness and a

Research Institute for Food Science, Kyoto University, Uji, Kyoto 611, Japan.

0021-8561/88/1436-0417$01.50/0 © 1988 American Chemical Society

hardness similar to that of the turbid gel prepared from nonproteolyzed ovalbumin, indicating that this proteolysis was the limited one. Usually pepsin does not show the limited proteolysis of protein, but in this study we showed that such limited proteolytic activity can be obtained at a given condition when ovalbumin and egg white were used as a substrate.

Pepsin is an acid protease that requires several subsites as well as a main binding site against good substrates (Fruton, 1976). From the studies of enzyme kinetics and chemical modification, the active site of pepsin is known to be composed of at least carboxylic groups, one (Asp-32, pH 1.5) protonated and the other (Asp-215, pH 4.5) unprotonated. The optimum pH for the hydrolysis of synthetic peptides varies between 2 and 4 depending on the structure of the peptides. Maximum rates for the hydrolysis of various proteins have been observed at pH 2. The preferential cleavage site for proteins may change at different pH regions. However, little evidence for the existence of different cleavage sites of the proteins at various pHs has been offered.

This paper describes the very limited proteolysis of ovalbumin by porcine pepsin observed at pH 4. Only a single peptide bond in the ovalbumin molecule was cleft, and a stable intermediate named *p*-ovalbumin, which gave a heat-induced transparent gel whereas the ovalbumin gave a turbid gel at the same heating condition, was obtained. This means that a slight hydrolysis of protein can result in a drastic change of the functional properties of food protein. The cleavage site was determined, and it is also discussed in terms of the amino acid sequence and the conformation of the ovalbumin.

MATERIALS AND METHODS

Ovalbumin was prepared by crystallization from fresh hen egg white in a half-saturated ammonium sulfate suspension. Crystallizations were done five times. Sodium azide (0.03%) and EDTA (0.1 mM) were added through the preparation of the ovalbumin and the following enzyme experiments to prevent microbial contamination and the probable oxidation of free SH groups. The ovalbumin was crystallized five times. The purified ovalbumin suspension was centrifuged and the precipitate collected. The precipitate was solubilized by the addition of a sodium phosphate buffer (20 mM, pH 7.5) containing sodium azide (0.03%) and EDTA (0.1 mM) and dialyzed against the same buffer. Concentration of ovalbumin was estimated from the absorbance at 280 nm with $E^{1\%}_{280\mathrm{nm}} = 0.712$ (Glazer et al., 1963).

Pepsin (from porcine stomach mucosa, 1:60 000) was obtained from Sigma Chemical Co. Carboxypeptidase Y purchased from the Oriental Yeast Co. was used to determine the carboxyl terminal of the protein. SDS–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (1970) with a minor modification (see legend in Figure 1).

Gel chromatography was carried out with Sephadex G-75 previously equilibrated with sodium phosphate buffer (20 mM, pH 7.5) containing sodium azide (0.02%): column size, 2 × 75 cm; flow rate, 15 mL/h; sample applied, 1 mL of the reaction mixture. The determination of the sulfhydryl group was performed by the procedure of Ellman (1959) with a minor modification. To 4.9 mL of sodium phosphate buffer (40 mM, pH 8.0), which contained SDS (0.48%), urea (8 M), and EDTA (1 mM), was added 0.1 mL of an ovalbumin solution (40 mg/mL). Native ovalbumin did not react with the Ellman's reagent without the addition of SDS and urea. The amino terminal of the protein was determined by the dansyl chloride method

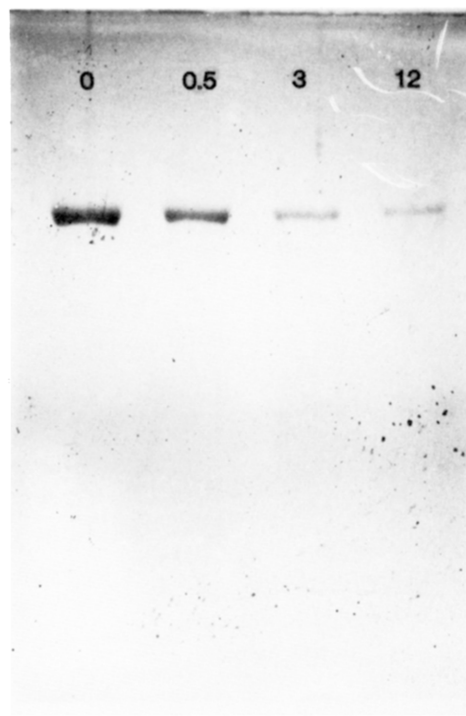

Figure 1. Proteolysis of ovalbumin by pepsin at pH 2.0, measured by SDS-PAGE. Reaction mixture (1 mL) contained 50 μL of ovalbumin (40 mg/mL), 4 μL of pepsin (10 mg/mL), 500 μL of hydrochloric acid–sodium acetate buffer (0.2 M, pH 2.0), and 10 μL of sodium azide (3 mg/mL). After incubation for the indicated period (numbers in the figure give reaction time in hours), 5 μL of 2-mercaptoethanol, 5 μL of bromophenol blue solution (0.05%), 40 μL of water, and 50 μL of the sample buffer for SDS-PAGE containing Tris buffer (125 mM, pH 7.0) and SDS (2%, w/v) were added to 20 μL of the reaction mixture and then heated for 3 min at 100 °C. Fifteen microliters of this mixture containing 3 μg of ovalbumin was applied to a SDS–polyacrylamide gel (13.5%, w/v). Migration occurred from top to bottom.

(Gray and Hartley, 1963). The dansylated protein was hydrolyzed with 5.7 M HCl for 20 h at 105 °C, and the dansyl amino acids were identified by two-dimensional thin-layer chromatography on polyamide sheets (7.5 × 7.5 cm).

The carboxyl terminal of the protein was determined by the use of carboxypeptidase Y followed by automatic amino acid analysis.

RESULTS

The enzymatic reaction of pepsin with ovalbumin (10 mg/mL) as the substrate was performed at various pHs (1–7) at 25 °C. Hydrochloric acid–sodium acetate buffer (pH 1–3), sodium acetate buffer (pH 4–5), and sodium phosphate buffer (pH 6–7) were used at 0.1 M concentrations. The hydrolysis of the ovalbumin molecule was judged by the change in the SDS-PAGE pattern. At pH 2 (Figure 1), the original band of the ovalbumin (MW 45 000) gradually diminished with time, almost disappearing after 12 h. No bands other than the original one appeared in the SDS-PAGE pattern, indicating that the ovalbumin molecule was hydrolyzed directly to small peptides. At pH 4 (Figure 2), the appearance of a new band (MW ≈ 42 000) was observed as the original band disappeared. After 12 h, all of the original ovalbumin molecule had become this smaller intermediate protein, which was stable against further reaction with pepsin. No hydrolysis was observed during further reaction for 12 h (lane 5 in Figure 2) or after the addition of more pepsin (lane 6 in Figure 2). This stable intermediate protein was named *p*-ovalbumin. At pH >5, no hydrolysis of the

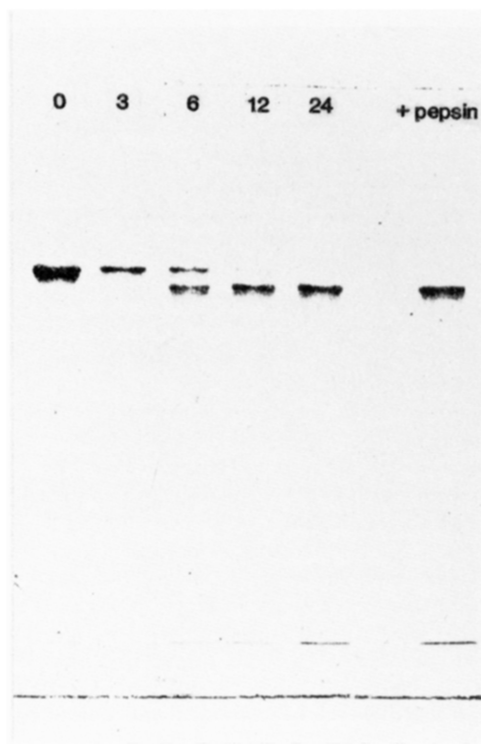

Figure 2. Proteolysis of ovalbumin by pepsin at pH 4.0, measured by SDS-PAGE. Details are the same as in Figure 1 except for pH. Numbers in the figure give the reaction time (hours), and "+ pepsin" means that the same amount of pepsin (40 μg/4 μL) was added again to the reaction mixture after 12-h incubation and the resultant mixture allowed to stand for 12 h.

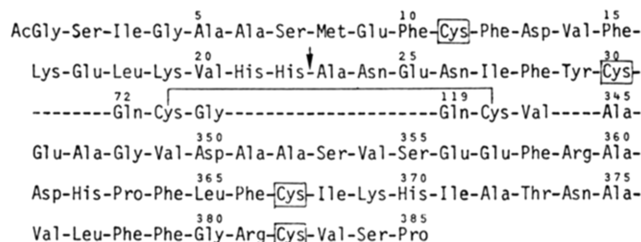

Figure 3. Primary amino acid sequence of ovalbumin. This sequence is given in Nisbet et al. (1982). The pepsin cleavage site at pH 4 is indicated by an arrow.

ovalbumin was observed. The above results show that the ovalbumin was hydrolyzed to the stable intermediate *p*-ovalbumin with a smaller molecular weight than the original ovalbumin, by pepsin at pH 4. A fragment of small molecular weight ($\approx$3000) released from the ovalbumin was not detectable by SDS-PAGE but was detected by gel filtration on Sephadex G-75. The hydrolysate of the ovalbumin at pH 4 by pepsin was applied on the Sephadex column. Two large peaks corresponding to *p*-ovalbumin and to this fragment and a minor peak corresponding to pepsin were observed on the chromatogram. The fractions containing *p*-ovalbumin or the fragment were collected and used for the analysis described below. The original ovalbumin was also treated on the same Sephadex column and used as a reference.

The amino acid sequence and the position of the S–S bridge have already been described by Nisbet et al. (1981) and are shown in Figure 3. The amount of free SH in the original ovalbumin and in the *p*-ovalbumin was determined by the use of Ellman's reagent.

The original ovalbumin contained 4.0 mol and the *p*-ovalbumin 3.2 mol of SH/mol of protein. The amount of SH in the ovalbumin found was consistent with that given

Table I. Release of Amino Acids from Ovalbumin or *p*-Ovalbumin by Carboxypeptidase Y Digestion[a]

ovalbumin	reactn time, min	Val	Ser	Pro
orig	5	0.19	0.38	0.72
OVA	20	0.57	0.63	0.95
p-OVA	5	0.28	0.45	0.54

[a] Six milligrams of native ovalbumin or *p*-ovalbumin in 2 mL of sodium phosphate buffer (20 mM, pH 7.5) was added to 0.1 mL of SDS solution (1.12 M) and the resultant mixture heated for 5 min at 90 °C. Then, 0.5 mL of *N*-ethylmorpholine acetate (1.0 M, pH 6.5) and 0.06 mL of carboxypeptidase Y (5 mg/mL), freshly prepared from powder, were added to the mixture. After incubation for 5 or 20 min at 25 °C, the enzyme reaction was stopped by addition of 0.5 mL of trichloroacetic acid solution (20%, w/v). The reaction mixture was concentrated on a rotary evaporator. The concentrate was mixed with 0.5 mL of citrate buffer (0.2 M, pH 2.2), and 0.3 mL of the mixture was put into an automatic amino acid analyzer (Hitachi Model 935). Each value represents moles of amino acid released per mole of protein.

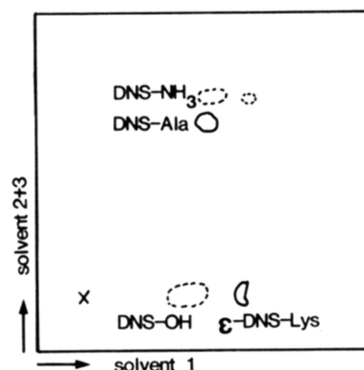

Figure 4. Thin-layer chromatography of dansyl amino acids on polyamide sheets. First, 80 μL of the *p*-ovalbumin (200 μg) obtained by G-75 Sephadex chromatography was added to 50 μL of redistilled *N*-ethylmorpholine, and 75 μL of dansyl chloride (25 mg/mL) was added to the mixture. After thorough mixing, the mixture was incubated for 1 h at 25 °C and 500 μL of acetone was added. The precipitate was collected by centrifugation (3000*g*, for 10 min), washed with 500 μL of 6 M HCl, and incubated for 18 h at 105 °C to hydrolyze the peptide. After incubation, the HCl was removed from the hydrolysate with NaOH pellets. The dried matter was solubilized by the addition of ethanol (10 μL) and spotted on polyamide layer sheets (7.5 × 7.5 cm, from Cheng Chin Trading Co., Ltd.). The first solvent was water–90% formic acid (200:3, v/v), the second solvent benzene–acetic acid (9:1, v/v), and the third solvent ethyl acetate–methanol–acetic acid (20:1:1, by volume).

by Nisbet et al. (1981). One cysteine residue was removed from the ovalbumin during this limited proteolysis by pepsin. Therefore, the site of the proteolytic cleavage must be either between Cys-11 and Cys-30 or between Cys-367 and Cys-382 (Figure 3).

The carboxyl terminal of the original ovalbumin and of the *p*-ovalbumin was determined with carboxypeptidase Y as shown in Table I. Proline, serine, and valine were successively released from the ovalbumin with time, as Nisbet et al. also reported (see Figure 3). In the case of *p*-ovalbumin, results similar to those for the original ovalbumin were obtained. This indicates that no cleavage occurred at the side close to the carboxyl terminal of the ovalbumin, that is between Cys-367 and Cys-382. The amino terminal of the original ovalbumin was not detachable by the *p*-ovalbumin as shown in Figure 4.

Spots corresponding to dansylalanine and dansyllysine were observed on the polyamide sheets. A minor spot was also found when the original ovalbumin was used, but that was not identified.

The results indicate that the amino terminal of *p*-ovalbumin is alanine. The only possible site of cleavage of the ovalbumin is the peptide bond between His-22 and Ala-23, which is between Cys-11 and Cys-30.

The amino and carboxyl terminals of the fragment (after 12 h of hydrolysis) separated by Sephadex G-75 were also examined as described above. No dansyl amino acids derived from the amino terminal of the fragment were detectable on the polyacrylamide sheets. The carboxyl terminal of the fragment was histidine. The results indicate that the fragment was a peptide from the amino terminal to the His-22 of the original ovalbumin. The fragment obtained after 24 h of hydrolysis contained the same carboxyl terminal (histidine). All these results indicate that only a single peptide bond (His-22–Ala-23) in the ovalbumin was cleft after reaction with pepsin at pH 4.

DISCUSSION

At pH 2.0, pepsin hydrolyzed the ovalbumin molecule into small peptides not detected by SDS-PAGE; that is, it attacked the many peptide bonds of ovalbumin at this pH.

However, at pH 4.0, pepsin acted on only one peptide bond in the ovalbumin molecule and produced a stable intermediate, *p*-ovalbumin.

The roughly estimated molecular weight of the released peptide (3000) from the SDS-PAGE pattern coincided well with the number of residues, determined by amino and carboxyl terminal analyses to be 22. Both the fragment peptide and the *p*-ovalbumin were resistant to further hydrolysis by pepsin at pH 4. The proteolysis might be limited for two reasons. One is the change of the substrate specificity of pepsin caused by changes in pH. The other is the conformation of the native ovalbumin.

As described in the introduction, two carboxyl groups in pepsin molecule are essential to its catalytic action. The enzyme is most active for various synthetic peptides between pH 1.5 and 4.5.

Most experiments for the hydrolysis of a protein substrate by pepsin are carried out at pH 1.8 or 2.0, but when the hydrolysis was carried out at pH 4 in this study, only a single peptide bond of the ovalbumin was hydrolyzed. In separate experiments, the hydrolysis of casein by pepsin was examined (data not shown). At pH 4, the formation of a intermediate protein (limited hydrolyzed ovalbumin) was observed on SDS-PAGE, indicating that, at least at pH 4, the cleavage sites of the protein were fewer than at pH 2. However, in the case of casein, the intermediate protein gradually diminished after prolonged incubation. This difference of ovalbumin and casein can be explained by the difference of their conformations. The casein molecule has a random structure at all pHs and does not need the addition of a denaturant even for proteolysis at neutral pH. However, most proteins need the addition of some denaturant or the treatment for denaturation of protein to be proteolyzed at neutral pH.

Ovalbumin is also resistant to proteolysis at neutral pH. For example, trypsin did not act at all at pH 7 (unpublished data). Subtilisin can hydrolyze two sites of ovalbumin (bonds 345–346 and 352–353).

At pH 4, the conformation of ovalbumin would not be very different from that at neutral pH, and the bonds between His-22 and Ala-23 are exposed at the surface, to be easily cleft by pepsin. Another peptide sequence between 345 and 353 is also probably exposed, as shown by experiments with subtilisin. However, this part of the peptide was not hydrolyzed at pH 4 by pepsin, probably by the decrease of cleavage sites by pepsin at pH 4.

It is known that ovalbumin is synthesized and secreted without the cleavage of a hydrophobic signal peptide, unlike other secretory proteins. Meek et al. (1982) speculated that the hydrophobic signal peptide lies between residues 25 and 45 and folds back toward the preceding residues to form an amphipathic hairpin structure. Residues 1–25 of ovalbumin do not possess a hydrophobic stretch, whereas residues 26–45 are all either hydrophobic or uncharged. They suggest that this amphipathic sequence forms one limb of a hairpin loop, which is the signal element responsible for interaction with the microsomal membrane. The site of pepsin attack is the curved part of the loop, that is, the conversion area from a hydrophilic amino acid sequence to a hydrophobic amino acid sequence. On the other hand, Lingappa et al. (1979) stated that the signal sequence involved in membrane translocation is located between residues 234 and 253.

Our studies suggest that His-22 and Ala-23 are exposed on the outside of the ovalbumin molecule and that when the hairpin loop is also on the outside and linked to the rest of the ovalbumin molecule (residue 50 linked to 385), pepsin will probably selectively attack only the peptide bond between His-22 and Ala-23. This supports the suggestion of Meek et al. (1982) rather than that of Lingappa et al. (1979).

Registry No. Pepsin, 9001-75-6; carboxypeptidase Y, 9046-67-7.

LITERATURE CITED

Adler-Nissen, J. *Enzymic Hydrolysis of Food Proteins*; Elsevier Applied Science: London, New York, 1986.

Ellman, G. L. "Tissue Sulfhydryl Groups". *Arch. Biol. Biophys.* **1959**, *82*, 70–77.

Fruton, J. S. "The Mechanism of the Catalytic Action of Pepsin and Related Acid Proteinases". *Adv. Enzymol.* **1976**, *44*, 1–36.

Glazer, A. N.; McKenzie, H. A.; Wake, R. G. "The Denaturation of Proteins II. Ultraviolet Absorption Spectra of Bovine Serum Albumin and Ovalbumin in Urea and in Acid Solution". *Biochim. Biophys. Acta* **1963**, *69*, 240–248.

Gray, W. R.; Hartley, B. S. "A Fluorescent End-group Reagent for Proteins and Peptides". *Biochem. J.* **1963**, *89*, 59–63.

Kay, J. "Proteolysis—A Degrading Business but Food for Thought". *Biochem. Soc. Trans.* **1982**, *10*, 277–280.

Kitabatake, N.; Doi, E. "Heat-Induced Transparent Gels Prepared from Pepsin Treated Ovalbumin and Egg White". *Agric. Biol. Chem.* **1985**, *49*, 2457–2458.

Laemmli, U. K. "Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4". *Nature (London)* **1970**, *227*, 680–685.

Lingappa, V. R.; Lingappa, J. R.; Blobel, G. "Chicken Ovalbumin Contains an Internal Signal Sequence". *Nature (London)* **1979**, *281*, 117–121.

Meek, R. L.; Walsh, K. A.; Palmiter, R. D. "The Signal Sequence of Ovalbumin Is Located Near the NH_2 Terminus". *J. Biol. Chem* **1982**, *257*, 12245–12251.

Nisbet, A. D.; Saudry, R. H.; Moir, A. J. G.; Fothergill, L. A.; Forthergill, J. E. "The Complete Amino-Acid Sequence of Hen Ovalbumin". *Eur. J. Biochem.* **1981**, *115*, 335–345.

Yamamoto, A. "Proteolytic Enzymes". In *Enzymes in Food Processing*; Reed, G., Ed.; Academic: New York, 1975.

Received for review September 2, 1987. Accepted December 28, 1987.