Characterization of Anti-Irradiation-Denatured Ovalbumin Monoclonal Antibodies. Immunochemical and Structural Analysis of Irradiation-Denatured Ovalbumin

Tetsuya Masuda,*,† Sakiyo-Yamaoka Koseki,‡ Kyoden Yasumoto,§ and Naofumi Kitabatake†

Research Institute for Food Science, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan, Department of Human Life Science, Seibo Jogakuin Junior College, Fukakusa, Fushimi-ku, Kyoto 612-0878, Japan, and Department of Food and Nutrition, Sugiyama Jogakuen University, Hoshigaokamotomachi, Chikusa-ku, Nagoya 464-8662, Japan

Five monoclonal antibodies (OVA-01, -02, -03, -04, -06) produced against irradiated ovalbumin were investigated in relation to the conformational change in the ovalbumin molecule induced by irradiation with Cobalt-60 γ-rays. Four antibodies (OVA-01, -02, -04, -06) recognized both native and irradiated ovalbumin, but OVA-03 reacted only with irradiated ovalbumin. These antibodies were classified by modified competitive ELISA, and their K_d values were determined by the Klotz equation. Epitope analyses were also performed on OVA-03 using CNBr-cleaved peptide fragments from ovalbumin, and it was confirmed that OVA-03 bound to the fragment corresponding to residues Val173-Met196 of the ovalbumin molecule that consists of internal β-sheet strand 3A and helix F1 containing one open turn. These results demonstrate that dramatic conformational changes in proteins can be induced or that some tertiary or secondary structures can be broken down by γ-ray irradiation, producing new antigenic sites.

Keywords: *Monoclonal antibody; ELISA; antigen−antibody interaction; ovalbumin; food irradiation*

INTRODUCTION

Food poisoning caused by pathogenic microorganisms such as *Salmonella enteritidis* and *Escherichia coli O157* can cause serious problems in our life (Imai et al., 1993; Kampelmacher, 1985; Roberts, 1989; Todd, 1989). γ-ray irradiation results in no toxicological hazard in any foods up to an overall average dose of 10 kGy, and it has therefore been widely used for food preservation in many countries (Griffith, 1992; Thakur and Singh, 1995). γ-ray irradiation is a physical process for treating foods, and as such, it is comparable to the heating or freezing, both of which are conventional methods of preservation. As with heating or freezing, γ-ray irradiation inhibits the sprouting of vegetables, delays the ripening of fruits and potatoes, kills insect pests in fruits and spices, and reduces or eliminates food-spoilage organisms such as salmonella (Clavero et al., 1994; Meng et al., 1998; Mitchell et al., 1991; Thakur and Arya, 1993; Thayer and Boyd, 1993; Wong et al., 1996). It is well-known that heating prevents the growth of organisms and also causes irreversible changes in protein structure; there is little information, however, regarding the effects of irradiation on protein structure, and still more remains to be elucidated as to what food constituents are damaged or which parts of protein structures are altered by irradiation. To detect irradiated products, a number of investigations have been performed. Most of these studies have focused on using physicochemical methods (Dodd et al., 1988; Sanderson et al., 1989; Stevenson et al., 1990; Stewart et al., 1993). However, it is necessary to evaluate the detection methods used for irradiated products from various viewpoints, for example biological, microbiological, and immunochemical.

Ovalbumin (OVA), a major globular protein of chicken egg white, is a phosphoglycoprotein with a molecular size of 45 kDa. OVA consists of a five-stranded β-sheet running parallel to the long axis of the molecule and an α-helix protruding as a loop that forms the reactive center. A number of studies on the structural (Huber and Carrell, 1989, Gettins et al., 1996, Huntington et al., 1997; Shirai et al., 1997), functional (Doi and Kitabatake, 1997), and immunological properties (Renz et al., 1993) of OVA have been performed. These investigations help us to describe the structural change or the antigenicity that occurs as a result of the denaturation process. Recently, antibodies have played a significant role in detecting the conformational features of proteins during the refolding or denaturation process. Collawn et al. (1988) have described antibodies with well-defined epitopes to detect conformational details that cannot be observed with conventional spectroscopic instruments, and Kaminogawa et al. (1989) have prepared antibodies against a bovine β-lactoglobulin and succeeded in monitoring the local conformational changes that occur during heat denaturation. Ikura et al. (1992) have identified the structural regions of the OVA molecule that change during heat denaturation.

In the present study, we prepared five monoclonal antibodies, OVA-01, -02, -03, -04, and -06, against irradiated ovalbumin and elucidated the specificity of

* To whom correspondence should be addressed. Tel: +81-774-38-3742. Fax: +81-774-38-3743. E-mail: masuda@food2.food.kyoto-u.ac.jp.

† Research Institute for Food Science, Kyoto University.

‡ Seibo Jogakuin Junior College.

§ Department of Food and Nutrition, Sugiyama Jogakuen University.

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each antibody by ELISA methods. To clarify the conformational changes of OVA induced by irradiation, epitope mapping of the I-OVA-specific antibody, OVA-03 was performed.

MATERIALS AND METHOD

Materials. OVA was purified from fresh egg white by crystallization in an ammonium sulfate solution (Sörensen and Hörup, 1915). After five recrystallizations, an extremely pure ovalbumin with homogeneous molecular weight as determined by SDS-polyacrylamide gel electrophoresis was obtained. Freund's complete adjuvant and Freund's incomplete adjuvant were from DIFCO (Detroit, MI). Pristane (2,6,10,14-tetramethyldecanoic acid) was from Wako Pure Chemical (Osaka, Japan). Cyanogen bromide (CNBr) was from Nacalai tesque (Kyoto, Japan). Poly(ethylene glycol) 4000 was from Merck (Darmstadt, Germany). Tissue culture plates and microtiter plates for ELISA were obtained from Becton Dickinson (Ontario, Canada). Blocking reagent for ELISA was from Boehringer Mannheim (Indianapolis, IN), and horseradish peroxidase (HRP) conjugated goat anti-mouse (H+L) was from ZYMED (San Francisco, CA). All other chemicals were of guaranteed reagent grade.

Preparation of Irradiated Ovalbumin (I-OVA). OVA was solubilized in 20 mM sodium phosphate buffer (pH 7.0), and aliquots were dialyzed against distilled water and lyophilized. This powder was irradiated in a Gamma cell 220 (4.7 × 10 Gy/h, 21 × 10 Tbq of ^{60}Co, Nordion international Inc., Canada) with Cobalt-60 γ-rays at a dose rate of 7.5 kGy/h with a dose of 100 kGy.

Preparation of Anti-Irradiated Ovalbumin Monoclonal Antibodies. Three mice were immunized with emulsion mixtures of I-OVA (30 μg) in phosphate-buffered saline (PBS) and Freund's complete adjuvant. Emulsions of I-OVA and Freund's incomplete adjuvant were intraperitoneally injected into BALB/c mice every 3 weeks. Finally, I-OVA (30 μg) in PBS containing no adjuvant was injected intravenously into the tail vein. Three days later, myeloma cells (P3 × 63-Ag.6.5.3.; Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) were fused with spleen cells from immunized mice at a ratio of 1:4, using 50% poly(ethylene glycol) 4000. Ten days after the fusion, culture supernatant was assayed by ELISA as described below. Positive cells were cloned by repeated limiting dilution and then injected intraperitoneally into mice previously primed with pristane. Ascitic fluids were precipitated by saturated ammonium sulfate solution and dissolved in 20 mM sodium phosphate buffer, pH 7.0. These crude antibodies were purified by Protein G affinity chromatography.

Determination of the Subclass of Antibodies. The subclass of antibodies was determined by double diffusion test using the MONOCLONAL ANTIBODY TYPINGKIT (University of Birmingham Research Institute).

ELISA Analysis. A 100 μL portion of I-OVA or N-OVA solution (10 μg /mL) in PBS was transferred into the wells of a microtiter plate and incubated overnight at 4 °C. The wells were washed with PBS containing 0.05% (v/v) Tween 20 (T-PBS) and then blocked by gelatin for ELISA (2.7 g/100 mL distilled water; Boehringer Mannheim). After washing with T-PBS, the culture supernatant of hybridomas was poured into the wells and incubated for 1.5 h at 37 °C. After washing, 100 μL of horseradish peroxidase (HRP) conjugated goat anti-mouse (H+L) at a final dilution of 1:3000 was added and incubated for 1.5 h. After the solution was discarded, bound secondary antibody was quantified by the addition of a substrate solution (10 mL of 50 mM sodium citrate buffer, pH 5.0 containing 4 mg of *o*-phenylenediamine and 2 μL of 30% H_2O_2). After 10 min incubation at 37 °C, the enzyme reaction was terminated by the addition of 50 μL of 2 N H_2SO_4. The absorbance was measured at 492 nm with an immunoreader MPR 4i (TOSOH, Japan).

Competitive ELISA Procedure. Antigen (N-OVA or I-OVA) in a 3-fold dilution series (competitor) was put together with antibody at a fixed concentration (10 or 1 μg/mL) and incubated overnight at 4 °C. A 100 μL portion of this incubated solution was picked out and transferred into another antigen-coated plate and then incubated overnight at 4 °C. The wells were washed with T-PBS, and HRP-labeled antibody was added. After this step, the ELISA procedure described above was carried out.

Procedure for the Modified Competitive ELISA. Antibody solution (Ab1; approximately 10 μg/mL, OVA-01, -02, -04, or -06) in 0.1 M $NaHCO_3$, pH 9.6, was added to the wells of the plates and incubated at 4 °C overnight. After washing with T-PBS, the wells were coated with blocking solution. After the solution was discarded, an excess amount of N-OVA (Ag) was added, mixed well, and incubated at 4 °C overnight (Ab1-Ag: binary complex). After removal of unbound N-OVA by washing with T-PBS, antibody solution (Ab2; approximately 10 μg/mL, OVA-01, -02, -04, or -06) diluted twice with T-PBS containing 10% blocking solution was added to these plates and incubated at 4 °C overnight. If the epitopes of each antibody are mutually different parts of the molecule, tertiary complexes (Ab1-Ag-Ab2) could be formed. On the contrary, the epitopes recognized by antibodies are overlapping, and tertiary complexes cannot be formed (unbound Ab2 was remained in the well). The solutions in the wells (unbound Ab2) were, then, transferred to new plate wells that had already been coated with N-OVA or I-OVA and quantified by the ELISA method described above. As a control, the same amount of each antibody (Ab2) was also added to the N-OVA or I-OVA coated plates and incubated in the same way. The difference in absorbance between the two plates corresponded to the amount of tertiary complexes (Ab1-Ag-Ab2).

Determination of K_d by the Klotz Equation. To elucidate the dissociation constant (K_d), we use the Klotz equation

$$A_0/(A_0 - A_n) = K_d/[Ag] + 1$$

where A_0 is the absorbance measured for the total antibody concentration in the absence of antigen and A_n is the absorbance measured in the presence of the antigen. By plotting $A_0/(A_0 - A_n)$ versus 1/[Ag], the slope of the straight line is equal to the K_d.

Epitope Map of Irradiation-Specific OVA-03 Antibody. Fragmentation of OVA was performed using cyanogen bromide (CNBr) with the method described by Nisbet et al. (1981). Western blot analysis of these fragments using OVA-03 was performed. Briefly, fragments of OVA were analyzed by SDS-PAGE according to the procedure of Laemmli (1970) and then electrotransferred onto nitrocellulose membrane using a Trans-Blot apparatus (Biorad). The membrane was soaked in the casein solution for 1 h. After being washed with 50 mM Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl, the membrane was incubated with OVA-03 (10 μg/mL). After washing with the same buffer, the membrane was incubated with H_2O_2 and 4-chlolo-1-naphthol (4-CN Substrate Kit, VECTOR, Burlingame, CA). The band recognized by the antibody was cut from the gel and purified by high-performance liquid chromatography systems (LC-6A, Shimadzu, Kyoto, Japan) using a reversed-phase column (5C18-AR−II, 4.6 × 150 mm, Nacalai tesque, Kyoto, Japan), and eluted in a linear gradient from 10% to 80% acetonitrile containing 0.1% trifluoroacetic acid (pH 2.0) at a flow rate of 1.0 mL/min. The fragments purified by HPLC were concentrated, and the primary sequence analysis was carried out on a gas-phase protein sequencer (model 477A/120A, Applied Biosystems).

RESULTS AND DISCUSSION

We obtained five monoclonal antibodies against I-OVA, and the properties of each antibody are shown in Table 1. The antibodies all belonged to the IgG1-type subclass. The specificity of these monoclonal antibodies was determined by competitive ELISA (Figure 1). Four antibodies reacted with both N-OVA and I-OVA, while the fifth, OVA-03, reacted only with I-OVA. The K_d values determined by the Klotz equation (Figure 2) are

Table 1. Properties of Anti-Irradiated OVA Antibodies. Specificity and K_d Value Were Determined by Competitive ELISA Shown in Figure 1[a]

antibody	specificity	K_d for N-OVA (10^{-7} M^{-1})	K_d for I-OVA (10^{-7} M^{-1})
OVA-01	both N- and I-OVA	1.5	3.0
OVA-02	both N- and I-OVA	0.5	2.6
OVA-03	only I-OVA	*b*	2.3
OVA-04	both N and I-OVA	4.6	4.8
OVA-06	both N and I-OVA	2.9	3.5

[a] The procedure of this assay was described in the Materials and Methods. K_d value against N-OVA or I-OVA was calculated by the Klotz equation shown in the text. [b] Not detected.

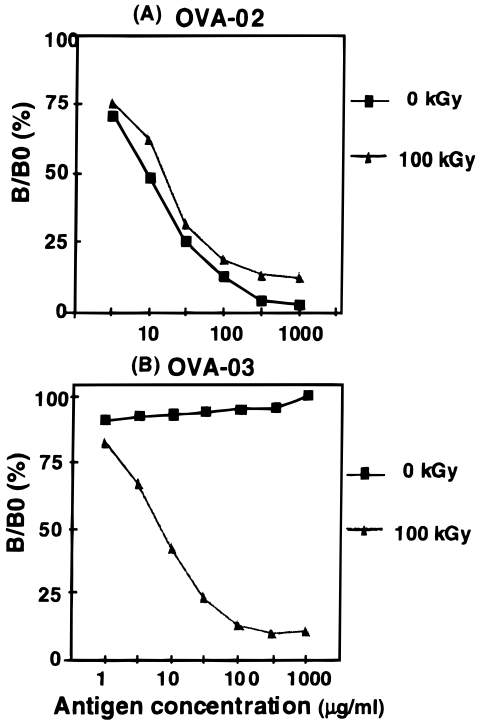

Figure 1. Binding curves of anti-irradiated ovalbumin monoclonal antibodies against 0 (N-OVA) and 100 kGy-irradiated ovalbumin (I-OVA). (A) represents OVA-02, and (B) represents OVA-03 antibody. Experiments followed the procedure for competitive ELISA (details provided in Materials and Methods). B/B_0 means (absorbance at the last steps of ELISA in the presence of various concentrations of antigen)/(absorbance in absence of a competitive antigen).

also shown in Table 1. OVA-01, -02, and -06 bind with slightly higher affinity to N-OVA, but in OVA-04 there

Table 2. Reactivities of Monoclonal Antibodies with Modified Competitive ELISA[a]

	Ab2				
Ab1	OVA-01	OVA-02	OVA-03	OVA-04	OVA-06
OVA-01	S	S	*b*	D	D
OVA-02	S	S	*b*	D	D
OVA-04	D	D	*b*	S	D
OVA-06	D	D	*b*	D	S

[a] When Ab1-Ag-Ab2 tertiary complex is formed, Ab1 and Ab2 have a different epitope. In this case, marked D. On the other hand, when Ab1-Ag-Ab2 tertiary complex is not formed, this result means Ab1 and Ab2 have an overlapping epitope or a same epitope marked S. [b] No reaction.

is little difference between N-OVA and I-OVA, and OVA-03 does not react with N-OVA at all. The modified competitive ELISA indicated that epitopes recognized by OVA-01 and OVA-02 are overlapping, but that OVA-01, OVA-04, and OVA-06 mutually recognize different parts of the epitope. On the other hand, OVA-03 did not react with the complex of antibody and N-OVA (Table 2). Just as misfolded heat-denatured OVA tended to rapidly aggregate in the presence of salt through hydrophobic interaction, I-OVA tended to self-aggregate, covering the epitopes to which the antibody was accessible in its native structure. In addition, N-OVA is known to be susceptible to surface denaturation, including denaturation by interactions with the surface of plastic plate (Varshney et al., 1991). Because modified competitive ELISA enables us to directly investigate the antibody−antigen interaction under liquid conditions, no modifications such as alkaline phosphatase labeling or radioisotope labeling of proteins is required. For this reason, we can mostly neglect the effects of the denaturation of antigens binding to the plate, and would be useful and applicable to study the local conformational changes in protein molecules or the process of the denaturation.

Next we attempted to determine the epitope of irradiated OVA specific antibody, OVA-03 for the reactivity of the CNBr fragments of OVA. I-OVA was degraded and aggregated by irradiation, producing many unknown fragments, and the heterogeneity of the fragments and the difficulty in separating the reactive fragments from I-OVA, making I-OVA unsuitable for direct epitope analysis. The CNBr fragments of OVA were separated by SDS−PAGE and some of them were reacted with OVA-03. The smallest reactive band of OVA-03 was cut from the gel and concentrated for the

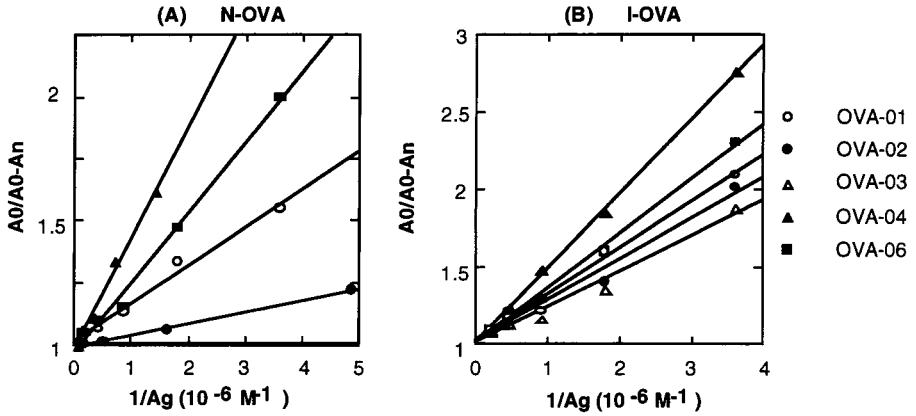

Figure 2. Klotz plot of the antibodies OVA-01 (○), OVA-02 (●), OVA-03 (△), OVA-04 (▲), and OVA-06 (■), to the N-OVA (A) and I-OVA (B). The slope of the straight line is equal to the K_d. A_0 is the absorbance measured for the total antibody concentration ([Abt]) in the absence of antigen. A_n is the absorbance measured in the presence of the antigen.

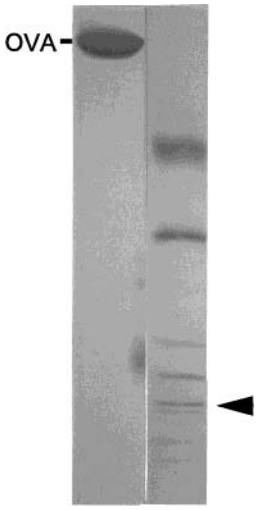

Figure 3. Western blot of CNBr-cleaved fragments: lane 1, heat-denatured ovalbumin; lane 2, CNBr-cleaved ovalbumin. The band indicated by an arrow was cut for the determination of the amino acid sequence.

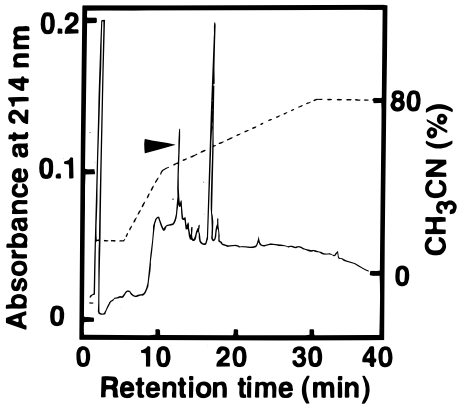

Figure 4. HPLC pattern of CNBr-cleaved fragments. CNBr-fragments were eluted in a linear gradient from 10% to 80% acetonitrile, containing 0.1% trifluoroacetic acids (pH 2.0) at a flow rate of 1.0 mL/min. The peak indicated by an arrow indicates a strong affinity to OVA-03 as shown by competitive ELISA. This peak was concentrated and analyzed by a gas-phase protein sequencer.

determination of the epitope (Figure 3). This concentrate was applied to a reversed-phase column. The elution pattern is shown in Figure 4. The peak indicated by an arrow was concentrated and analyzed by a gas-phase protein sequencer, and five amino acid residues were determined: Val-Leu-Val-Asn-Ala. This sequence corresponds to Val173-Ala177 primary sequence (Figure 5) of N-OVA, which is a part of the central β-strand 3A (172-MVLVNAIVFKGLWEK-186). Strand 3A in N-OVA represents the spine of the molecule and is covered by both helix F (Ala138-Gln152) and the turn between helix F, and strand 3A (Gln152-Met172), which is antiparallel to helix F, forms a sequence of bulges (Huber and Carrell, 1989). The residues in this segment are abundant with aliphatic and hydrophobic amino acids. They are positioned internally in the molecule and are tightly packed or close to other conserved residues. As OVA-03 does not react with N-OVA at all, we can conclude that irradiation causes conformational changes in helix F and the turn that protects strand 3A, and the newly antigenic sites are then exposed on the molecular surfaces. To confirm whether this conformational change is due only to irradiation, we also investigated heat-

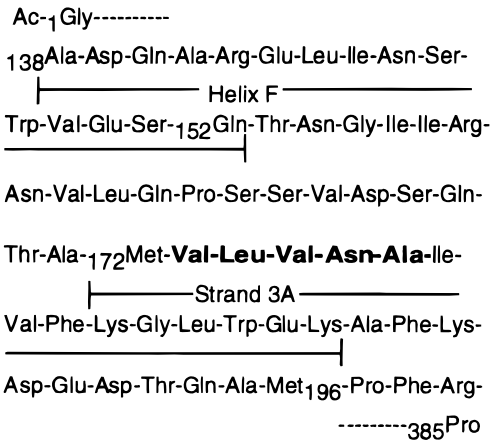

Figure 5. Amino acid sequence of ovalbumin. The underlined sequence represents the region that contains the epitope of OVA-03. The primary structure of ovalbumin is that reported by Nisbet et al., and the secondary structures, helix F and strand 3A, are assigned according to Stein et al. (1990). The bold regions (Val-Leu-Val-Asn-Ala) were determined by the protein sequencer.

denatured, urea-denatured, and guanidine-denatured OVA using the OVA-03 conformationally sensitive antibodies as probes (data not shown). It was found that the conformational changes and the exposure of newly antigenic sites occurs not only due to irradiation, but also in response to heat, urea, and guanidine denaturation. These results indicate that the denaturation of N-OVA is primarily responsible for the conformational change of helix F and the subsequent turn covering the epitope of OVA-03. In fact Asn149 and Thr156 in helix F form hydrogen bonds with the subsequent turn segment and may play a crucial role in the stabilization of this segment. By destabilizing the hydrogen bonds between helix F and the turn segment, strand 3A would be readily exposed to the molecular surface, and newly antigenic sites would be produced.

Though further detailed studies are necessary to elucidate the antigenicity caused by irradiation, the five monoclonal antibodies obtained in this study are useful tools, not only in understanding the effect of irradiation, but also in investigating in detail the denaturation process of OVA.

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LITERATURE CITED

Clavero, M. R.; Monk, J. D.; Beuchat, L. R.; Doyle, M. P.; Brackett, R. E. Inactivation of *Escherichia coli* O157: H7, salmonellae, and Campylobacter jejuni in raw ground beef by gamma irradiation. *Appl. Environ. Microbiol.* **1994**, *60*, 2069.

Collawn, J. F.; Wallace, C. J. A.; Proudfoot, A. E. I.; Paterson, Y. Monoclonal antibodies as probes of conformational changes in protein engineered cytochrome *c*. *J. Biol. Chem*. **1988**, *263*, 8625.

Dodd, N. J.; Lea, J. S.; Swallow, A. J. ESR detection of irradiated food. *Nature* **1988**, *334*, 387.

Doi, E.; Kitabatake, N. In *Food proteins and their applications*, *Structure and functionality of egg proteins*; Damodaran, S., Paraf, A., Eds.; Marcel Dekker: New York, 1997; pp 325.

Gettins, P. G. W.; Patston, P. A.; Olson, S. T. In *Serpins*: *Structure, function and biology*; R. G. Landes Co.: Austin, TX, 1996.

Griffith, G. Irradiated foods: new technology, old debate. *Trends Food Sci. Technol.* **1992**, *3*, 251.

Huber, R.; Carrell, R. W. Implications of the three-dimensional structure of α1-antitrypsin for structure and function of serpins. *Biochemistry* **1989**, *8*, 8951.

Huntington, J. A.; Fan, B.; Karlsson, K. E.; Deinum, J.; Lawrence, D. A.; Gettins, P. G. W. Serpin conformational change in ovalbumin. Enhanced reactive center loop insertion through hinge region mutations. *Biochemistry* **1997**, *36*, 5432.

Ikura, K.; Higashiguchi, F.; Kitabatake, N.; Doi, E.; Narita, H.; Sasaki, R. Thermally induced epitope regions of ovalbumin identified with monoclonal antibodies. *J. Food Sci.* **1992**, *57*, 635.

Imai, C.; Kurihara, K.; Umezawa, T. Egg and Salmonella. *New Food Indust. Jpn.* **1993**, *35*(1), 81.

Kaminogawa, S.; Shimizu, M.; Ametani, A.; Hattori, M.; Ando, O.; Hachimura, S.; Nakamura, Y.; Totsuka, M.; Yamauchi, K. Monoclonal antibodies as probes for monitoring the denaturation process of bovine β-lactoglobulin. *Biochim. Biophys. Acta* **1989**, *998*, 50.

Kampelmacher, E. H. Benefits of radiation processing to public health. *Radiat. Phys. Chem.* **1985**, *25*, 201.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680.

Meng, J.; Zhao, S.; Doyle, MP.; Joseph, S. W. Antibiotic resistance of *Escherichia coli* O157:H7 and O157:NM isolated from animals, food, and humans. *J. Food Prot.* **1998**, *61*, 1511.

Mitchell, G. E.; Isaacs, A. R.; Williams, D. J.; McLauchlan, R. L.; Nottingham, S. M.; Hammerton, K. Low dose irradiation influence on yield and quality of fruit juice. *J. Food Sci.* **1991**, *56*, 1628.

Nisbet, A. D.; Saundry, R. H.; Moir, A. J. G.; Fothergill, L. A.; Fothergill, J. E. The complete amino acid sequence of hen ovalbumin. *Eur. J. Biochem.* **1981**, *115*, 335.

Renz, H.; Bradley, K.; Larsen, G. L.; McCall, C.; Gelfand, E. W. Comparison of the allergenicity of ovalbumin and ovalbumin peptide 323−339 Differential expansion of Vβ-expressing T cell populations. *J. Immunol.* **1993**, *151*, 7206.

Roberts, T. Human illness costs of foodborne bacteria. *Am. J. Agric. Econ.* **1989**, *71*, 468.

Sanderson, D. C. W.; Slater, C.; Cairns, K. J. Detection of irradiated food. *Nature* **1989**, *340*, 23.

Shirai, N.; Tani, F.; Higasa, T.; Yasumoto, K. Linear polymerization caused by the defective folding of a noninhibitory serpin ovalbumin. *J. Biochem. (Tokyo)* **1997**, *121*, 787.

Sörensen, S. P. L.; Hörup, M. Studies on proteins. I. On the preparation of egg-albumin solutions of well-defined composition, and on the analytical methods used. *Compt. Rend. Trav. Lab. Carlsberg.* **1915**, *12*, 12.

Stein, P. E.; Leslie, A. G. W.; Finch, J. T.; Turnell, W. G.; McLaughlin, P. J.; Carrell, R. W. Crystal structure of ovalbumin as a model for the reactive centre of serpins. *Nature* **1990**, *347*, 99.

Stevenson, M. H.; Crone, A. V. J.; Hamilton, J. T. G. Irradiation detection. *Nature* **1990**, *344*, 202.

Stewart, E. M.; Stevenson, M. H.; Gray, R. The effect of irradiation dose and storage time on the ESR signal in the cuticle of different components of the exoskeleton of Norway lobster (Nephrops norvegicus). *Appl. Radiat. Isot.* **1993**, *44*, 433.

Thakur, B. R.; Arya, S. S. Effect of sorbic acid on irradiation induced sensory and chemical changes in sweetened orange juice and mango pulp. *Int. J. Food Sci. Technol.* **1993**, *28*, 371.

Thakur, B. R.; Singh, R. K. Combination processes in food irradiation. *Trends Food Sci. Technol.* **1995**, *6*, 7.

Thayer, D. W.; Boyd, G. Elimination of *Escherichia coli* O157: H7 in meats by gamma irradiation. *Appl. Environ. Microbiol.* **1993**, *59*, 1030.

Todd, E. C. D. Preliminary estimates of cost of foodborne disease in the United States. *J. Food Prot.* **1989**, *52*, 595.

Varshney, G. C.; Mahana, W.; Filloux, A. M.; Venien, A.; Paraf, A. Structure of native and heat-denatured ovalbumin as revealed by monoclonal antibodies: Epitopic changes during heat treatment. *J. Food Sci.* **1991**, *56*, 224.

Wong, Y. C.; Herald, T. J.; Hachmeister, K. A. Comparison between irradiated and thermally pasteurized liquid egg white on functional, physical, and microbiological properties. *Poult. Sci.* **1996**, *75*, 803.

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