JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2008,** *56,* 1727–1733 **1727**

In Vitro Determination of the Allergenic Potential of Technologically Altered Hen's Egg

SABINE HILDEBRANDT,[†] HARTMUT D. KRATZIN,[§] RAPHAËL SCHALLER,[#] RODOLPHE FRITSCHÉ,[#] HANS STEINHART,[†] AND ANGELIKA PASCHKE*,[†]

Food Chemistry, Faculty of Mathematics, Informatics and Natural Sciences, University of Hamburg, Grindelallee 117, D-20146 Hamburg, Germany; AG Proteomics, Max-Planck-Institute of Experimental Medicine, Hermann-Rein-Strasse 3, D-37075 Göttingen, Germany; and Nestlé Research Center, P.O. Box 44, 1000 Lausanne 26, Switzerland

Hen's egg allergy represents one of the most common and severe IgE-mediated reactions to food in infants and young children. It persists, however, in many cases also lifelong. Therefore, the aim of this study was the detailed analysis of a technological process used to reduce the allergenic potential of hen's egg. The investigation focused on the pasteurized egg as starting material, intermediate, and final products of a nine-step manufacturing process performed for use of eggs in convenience products appropriate for allergic individuals. The steps consisted of a combination of various heat treatments and enzymatic hydrolyses. The alterations were controlled by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting, enzyme allergosorbent test (EAST) inhibition, and mass spectrometry. Thereby it could be demonstrated that the allergenic potential of the raw material was reduced from step to step, and despite the known stability against heat and proteolysis of certain egg proteins, the total allergenic potential was finally below $^1/_{100}$ that of the starting material without a significant change in texture and flavor as evaluated in various products.

KEYWORDS: Hen's egg; heat treatment; enzymatic hydrolysis; food allergy; allergenic potential

INTRODUCTION

Hen's egg is one of the most frequent causes of adverse reactions to food in children, and sometimes this allergy is retained in adult life (*1–4*). Major allergenic egg proteins are ovalbumin (Gal d 2), conalbumin (Gal d 3), ovomucoid (Gal d 1), and lysozyme (Gal d 4). At least 24 antigenic hen's egg components are known (*5*). Allergologically significant are mainly the fractions of ovomucoid, ovalbumin, ovotransferrin (respectively, conalbumin) and lysozyme. These proteins make up 80% of the total protein content of egg white. The rest are, in the case of food allergy, less significant proteins such as macroglobulin, avidine, and several different enzymes. Within the scope of the European Union project REDALL (Reduced Allergenicity of Processed Food, QLK1-CT-2002-02687), industrial technologies are used and tested for the production of commercial products using hen's egg in order to make them accessible also for the allergic consumer. Despite various procedures normally used for food processing, the allergenicity of hen's egg could not be reduced to a level that is suitable for allergic people under preservation of the desired properties (texture and flavor) of hen's egg.

Two treatments that have been used to influence the allergenicity of hen's egg have to be considered: thermal processing and enzymatic hydrolysis. An important factor for the outcome of thermal processing is the raw material itself. Fresh egg white has a pH of 7.6–7.9. After storage, the pH rises up to 9.7. Therefore, it is important to control the age of hen's egg before heat is applied. The shift to alkaline conditions during storage may influence the heat-induced hydrolysis during the process. Furthermore, thermal processing may generate disulfide-linked polymers with unwanted properties due to disulfide interchange, a reaction that is catalyzed by alkaline conditions. Therefore, the age of the eggs used is crucial.

It has been shown that enzymatic hydrolysis of hen's egg proteins reduces the allergenicity of various proteins efficiently (*6–9*). This is enhanced if the proteins are partially or fully denatured because denaturation renders proteins more accessible to enzymatic digestion and different epitopes will be affected. The enzymes used to reduce the allergenic potential of hen's egg will cleave the egg proteins better after heat denaturation, that is, pasteurization or other thermal processing. Because smaller fragments are produced, linear epitopes can be destroyed. Epitopes, however, which were hidden in the native material, may be generated by heat denaturation.

* Author to whom correspondence should be addressed (telephone +49 40 42838 4353; fax +49 40 42838 4342; e-mail angelika.paschke@uni-hamburg.de).

[†] University of Hamburg.

[§] Max-Planck-Institute of Experimental Medicine.

[#] Nestlé Research Center.

10.1021/jf0725981 CCC: $40.75
Published on Web 02/09/2008

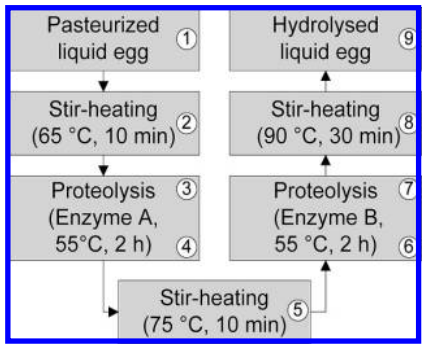

Figure 1. Steps of the technological process.

The above findings have been shown mostly for purified egg proteins. Because industrial production handles the complex egg matrix, all proteins were considered and a pooled serum mixed from 11 individuals allergic to hen's egg was used to evaluate a process that has been designed to reduce the allergenic potential of hen's egg. To reach the desired effect, a combination of both thermal processing and enzymatic hydrolysis was applied.

MATERIALS AND METHODS

Reagents. Phosphate-buffered saline (PBS, 150 mM NaCl, 10 mM K_2HPO_4 at pH 7.4) was prepared as described by Bernhisel-Broadbent et al. (*1*). If not otherwise mentioned, all chemicals were of analytical grade.

Raw Material. The starting material was pasteurized liquid whole egg, FT/OVO/0105 R, ABCD S.A., Avicole Bretonne Cecab Distribution (Ploërmel, France).

Technological Process. The various processing steps of the technologically altered egg samples are demonstrated in **Figure 1**. The conditions for initial pasteurization of liquid eggs are 67 ± 2 °C during 6 min. Liquid whole egg was heated at 65 °C for 10 min with stirring at 250 rpm. After the egg had cooled to 55 °C, 5% of Protamex (Enzyme A, Novozymes, Bagsvaerd, Denmark) was added, and the mixture was maintained at 55 °C for 2 h. After this first hydrolysis step, the mixture was heated at 75 °C for 10 min. The mixture was then cooled to 55 °C, 5% of Flavourzyme (Enzyme B, Novozymes) was added, and the mixture was maintained at 55 °C for 2 h. After this second hydrolysis step, the mixture was heated at 90 °C for 30 min. Sampling during the steps of proteolysis was performed after 1 h and after 2 h, respectively. All samples were frozen and stored at −20 °C.

Patient Sera. Sera were collected from 11 patients with egg allergy and a positive EAST (Spez. IgE ELISA RV 5, Allergopharma, Reinbek, Germany), class 2−5 for egg white, and pooled. Patients were procured by the Technical University of Munich (Department of Dermatology and Allergology), the University Hospital of Zurich (Allergiestation, Dermatologische Klinik), the Macedonio Melloni Hospital of Milan (Department of Pediatrics), and the Medical University of Vienna (Department of Pediatrics and Juvenile Medicine).

Protein Determination. Protein concentrations were determined relatively according to the method of Bradford (*10*) using bovine serum albumin as standard and Bradford reagent consisting of Coomassie Brilliant Blue G-250 and phosphoric acid.

Protein Extraction and SDS-PAGE. Protein extracts were carried out by mixing 1 g of the sample with 9 mL of PBS in a laboratory blender (Waring, New Hartford, CT) for 5 min. The homogenate was extracted for 1 h on a laboratory shaker (Bühler, Tübingen, Germany) at 4 °C and centrifuged for 30 min with 950*g* (Sigma, Osterode, Germany). The supernatant of extract 1 (see **Figure 1**) was diluted with PBS to a total protein concentration of 300 μg/mL and mixed 1:2 with loading buffer according to the method of Vieths et al. (*11*). The supernatants of extracts 2–9 were diluted with the same volume ratio of PBS and loading buffer as extract 1, respectively. Denaturing SDS-PAGE was performed according to the method of Laemmli (*12*), using 4–12% *N*,*N*-methylenebisacrylamide tris[hydroxymethyl]aminomethane (BisTris), Nu-PAGE gels, and 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (Invitrogen, Karlsruhe, Germany). Proteins were identified with silver staining according to the method of Heukeshoven and Dernick (*13*) with a detection limit of 0.05–0.1 ng/mm^2.

Immunoblotting. After SDS-PAGE, allergens were identified by transferring the proteins to a nitrocellulose (NC) membrane (pore size = 0.2 μm, Schleicher & Schüll, Dassel, Germany) according to the method of Towbin and Gordon (*14*) and detecting them by immunostaining with patients' sera based on the method of Szépfalusi et al. (*15*).

EAST Inhibition. For EAST inhibition assay egg proteins from extract solution 1 were coupled to cyanogen bromide activated paper disks (Schleicher & Schüll) using a modified method from Ceska and Lundkvist (*16*). Fifty microliters of patients' serum pool (diluted 1:2), previously incubated with different concentrations of protein extract solutions (1–9), were subsequently added to the disks and incubated for 3 h at room temperature in cavities of a microtiter plate (Minisorb, 96 cavity, Nunc, Roskilde, Denmark). Allergopharma (Reinbek, Germany) test kit (Spez. IgE ELISA RV 5) was used for EAST inhibition according to the manufacturer's recommendations with modifications. Free binding sites were blocked with ethanolamine for 1 h. Dilution series of the inhibitor extracts (containing egg protein extracts 1–9) were prepared in seven steps (undiluted, 1:10, 1:100, 1:1000, 1:10000, 1:100000, 1:1000000). Potato protein was used to check nonspecific inhibition. A total of 50 μL of diluted pool serum was added and incubated for 1 h at 37 °C in the dark. After three washes with 1% Tween 20 in PBS, 50 μL of anti-human IgE alkaline phosphatase conjugate (Allergopharma, Reinbek, Germany, diluted 1:200 in incubation buffer) was added and incubated for 1.5 h at 37 °C in the dark. The plate was washed again, and the bound enzyme activity was stained with 200 μL of staining solution [containing *p*-nitrophenylphosphate (PNPP)] for 1 h at 37 °C in the dark. After the addition of stopping solution (100 μL, 1 M NaOH), absorbance was measured at 405 nm. All EAST inhibition experiments were performed in duplicate, and data are given in mean values.

Two-Dimensional (2D) Gel Electrophoresis. 2D gel electrophoresis was performed for protein separation with isoelectric focusing (IEF) over the p*I* range of 3–7 [IEF gel: Novex, pH 3–7, Invitrogen, Karlsruhe, Germany, IEF marker (IEFM): Serva Liquid Mix 3–7, Serva, Heidelberg, Germany] used for the first dimension and SDS-PAGE for the second dimension as described by Görg et al. (*17*). Gels were stained with colloidal Coomassie Brilliant Blue (CBB) according to the method of Lanne and Panfilov (*18*) with an additional immobilization step according to Neuhoff et al. (*19*). For this the gel was incubated with a fixation solution (12% trichloric acid) for 3 h at room temperature on a laboratory shaker (Bühler, Tübingen, Germany). After three washings with water for 20 min, respectively, the gel was incubated for 1 h with incubation solution (containing methanol, phosphoric acid and ammonium sulfate) and incubated again with fixation solution (containing ethanol and phosphoric acid) according to the method fo Neuhoff et al. (*19*). After incubation with incubation solution, 20 mg of CBB G-250 was added and incubated for 3−4 days and washed with water several times. The selected spots were manually excised and subjected to mass spectrometric analysis.

Protein Identification. Excised gel plugs were subjected to an automated platform for the identification of gel-separated proteins as recently described in detail (*20*). Briefly, the robotic liquid handling system Genesis ProTeam 150 Advanced Digest (Tecan) was used to perform the tryptic in-gel digest with prior reduction/carboxamidomethylation of the proteins and to subsequently prepare the extracted tryptic peptides for matrix-assisted laser desorption−ionization−time of flight−mass spectrometry (MALDI-TOF-MS) on prestructured sample supports (AnchorChip, Bruker Daltonics) according to the thin layer affinity method (*21*). Using an Ultraflex I MALDI-TOF/TOF mass spectrometer (Bruker Daltonics), peptide mass fingerprint (PMF) spectra were automatically acquired, postprocessed, and subjected to database searches as described (*20*). To confirm these results and to detect also proteins that are often difficult to identify by peptide mass fingerprinting such as proteins in mixtures, post-translationally modified proteins, and small proteins, the mass spectrometer was operated in the MS/MS mode within the same automated analysis loop to record fragment ion spectra

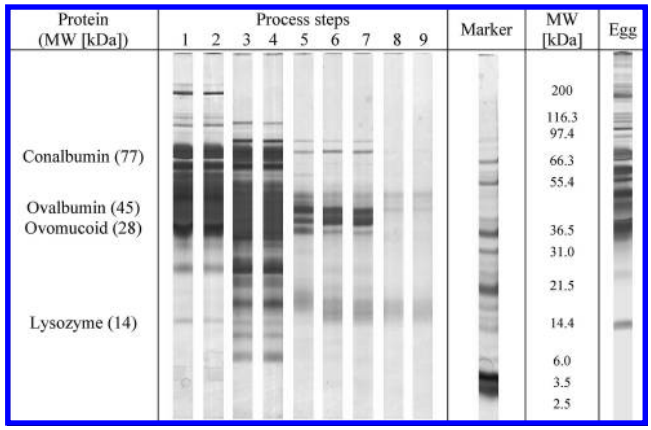

Figure 2. SDS−polyacrylamide gel of the nine process steps. Staining was done with silver. For comparison in lane "egg" the untreated egg proteins are shown.

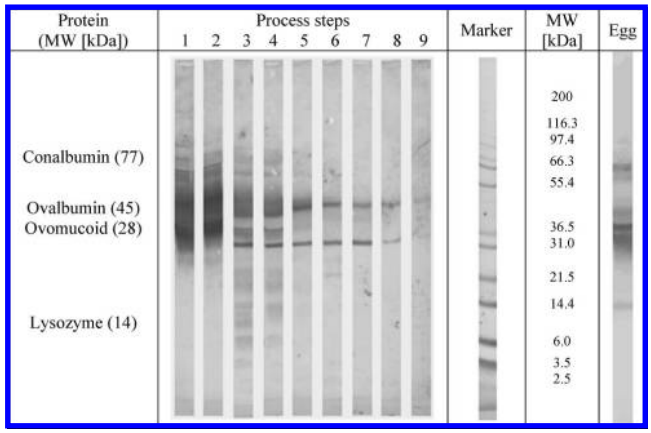

Figure 3. Immunoblot of the nine process steps.

of up to four selected precursor ions in a result-dependent manner. Database searches in the Swiss-Prot or NCBInr primary sequence database restricted to the taxonomy *Metazoa* were performed using the Mascot Software 2.0 (Matrix Science) licensed in-house. Carboxamidomethylation of cysteines was specified as fixed and oxidation of methionines as variable modification. The monoisotopic mass tolerance was set to 100 ppm, and one missed cleavage was allowed. Database searches of MS/MS data sets were performed as above with the fragment mass tolerance set to 0.7 Da. Only proteins represented by at least one peptide sequence above the significance threshold in combination with the presence of at least four peptide masses assigned in the PMF were considered to be identified.

RESULTS

Protein Content of Hen's Egg before, during, and after Industrial Processing. Egg proteins of the process steps were extracted from liquid material as described under Materials and Methods, the concentration was determined according to the Bradford assay (*10*), and equal amounts were loaded onto a 1D SDS-polyacrylamide gel. Visualization was done by silver staining (*13*) (**Figure 2**). Thereby in the untreated egg the most allergenic proteins of egg white were detected in the form of prominent bands. These are lysozyme (14 kDa), ovomucoid (28 kDa), ovalbumin (45 kDa), and conalbumin (also referred to as ovotransferrin, 77 kDa). Weaker bands represent vitellogenin (206 kDa) and other high molecular mass components not characterized here. Although ovomucoid and ovalbumin have different molecular masses, they cannot be separated completely by methods such as SDS-PAGE (*1*). This is due to N-linked carbohydrate residues (10 potential glycosylation sites, 5 at least

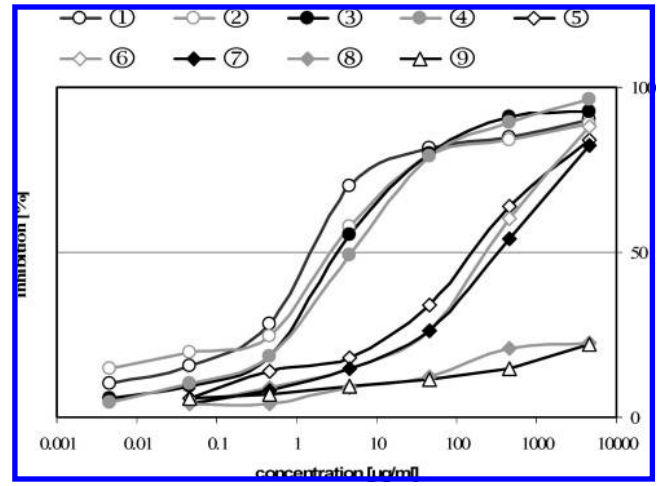

Figure 4. EAST inhibition with the nine process step samples as inhibitors.

Table 1. C_{50} Values of the EAST Inhibition Experiments

process step	C_{50} value (μg/mL)
1	2.6
2	3.7
3	4.0
4	5.9
5	270
6	340
7	403
8	–
9	–

partially modified), which considerably contribute to the molecular mass and lead to the well-known smear of glycoproteins and, in the case of ovomucoid, reduce the staining properties. This behavior leads to the incomplete separation of ovomucoid and ovalbumin. Furthermore, glycosylated tryptic peptides obtained after digestion cannot be identified by mass spectrometry because these masses are not stored in the databases. Hence, the number of tryptic fragments to be identified by mass spectrometry is reduced and identification is made more difficult (*22, 23*).

After steps 1 and 2 of the technological process, many bands are identical to the untreated material (lane "egg"). Only lysozyme is affected by the pasteurization process. A significant amount is destroyed. The stir-heating for 10 min at 65 °C seems to be of minor influence. The following protease treatment at 55 °C is obviously completed already after 1 h. Vitellogenin, ovalbumin, and ovomucoid are degraded to smaller fragments by the enzyme. This is obvious by the increase of bands at about 26, 18, 10, and 7 kDa. The predominant degradation of the 77, 45, and 28 kDa proteins, however, is achieved during stir-heating in step 5, whereas the second enzyme in steps 6 and 7 does not change the protein composition considerably. However, as already observed in step 5 the final protein degradation is achieved by the stir-heating at 90 °C. After 30 min, proteins were not detectable except for two faint bands at 45 and 16 kDa. This result resembles the hydrolyzed liquid egg shown in step 9.

Immunological Characterization of Hen's Egg Allergens. To evaluate the allergenic potential remaining in the processed liquid eggs, an SDS-polyacrylamide gel identical to that described above was prepared and the proteins of the nine extracts were transferred to a nitrocellulose membrane according to the method of Towbin and Gordon (*14*). To get an overall

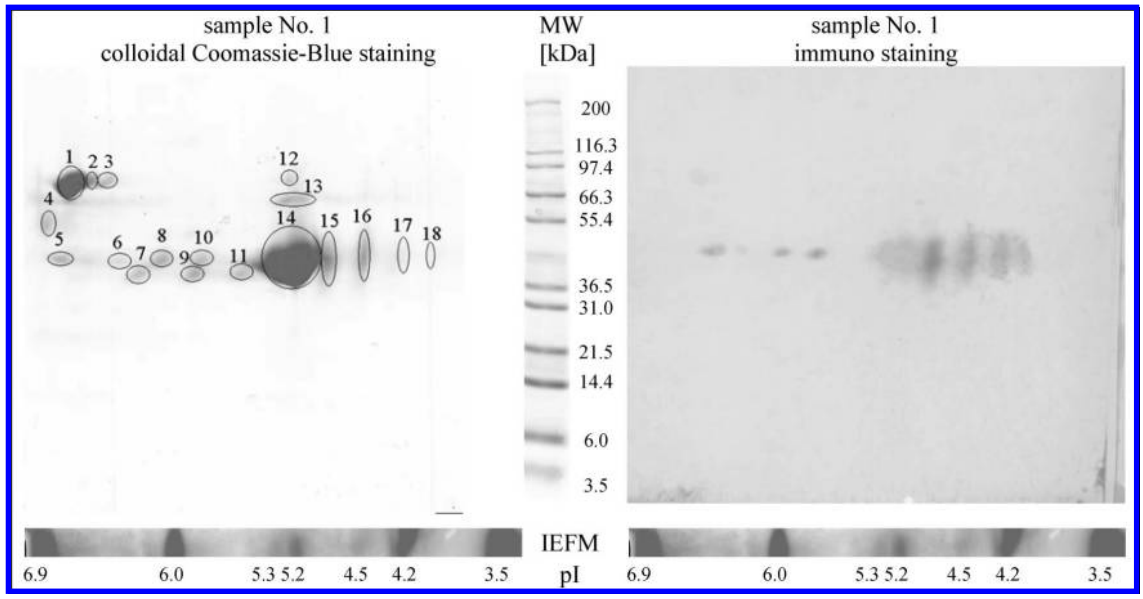

Figure 5. 2D electrophoresis of process step 1 with the corresponding immunoblot. Numbers indicate samples investigated by MALDI-MS (see **Table 2**).

Table 2. Mass Spectrometric Identification of Proteins from Process Step 1 Separated by 2D Gel Electrophoresis[a]

	Mascot data						2D electrophoresis			
no.	MS score	SC (%)	MS/MS score	peptide[b] score	mass[c] (kDa)	pI[c]	mass[d] (kDa)	pI[d]	IB	protein
1	305	56	245	100/145	77.5	6.7	~78	~6.7	+	conalbumin
2	381	66	124	46/78	77.5	6.7	~78	~6.6	+	conalbumin
3	299	58	144	63/81	77.5	6.7	~78	~6.5	−	conalbumin
4	142	41	116	45/72	54.4	6.2	~54	~6.8	−	ovoinhibitor
5	120	51	225	129/96	43.1	5.2	~44	~6.7	+	ovalbumin
6	141	53	154	147/77	43.1	5.2	~44	~6.3	−	ovalbumin
7	−	−	157	54/65	206.9	9.2	~40	~6.2	−	vitellogenin[e]
8	−	−	−	−	−	−	~44	~6.1	+	−
9	−	−	206	57/79/70	206.9	9.2	~41	~5.8	−	vitellogenin[e]
10	−	−	127	57/70	206.9	9.2	~44	~5.7	+	vitellogenin
11	−	−	64	49/15	206.9	9.2	~42	~5.5	−	(vitellogenin)[e]
12	105	51	147	65/83	43.1	5.2	~78	~5.2	−	ovalbumin/ovotransferrin[e]
13	93	51	218	80/67/72	43.1	5.2	~65	~5.2	−	ovalbumin
14	206	69	213	105/108	43.1	5.2	~44	~5.2	+	ovalbumin/α-livetin[e]
15	−	−	288	119/169	21.2	4.8	~45	~4.8	+	ovomucoid
16	53	41	106	119/168	21.2	4.8	~45	~4.5	+	ovomucoid/ovalbumin[e]
17	52	48	288	155/169	21.2	4.8	~45	~4.2	+	ovomucoid
18	−	−	−	−	−	−	~45	~4.0	+	−

[a] Score values for peptide mass fingerprint (MS Score) and peptide fragment fingerprint (MS/MS-Score) as well as single peptide scores and sequence coverage (S.C.) are as determined by the Mascot algorithm. [b] Values for different peptides are separated by (/) and have a significance threshold of 41−44 (larger values represent identity). [c] Masses and isoelectric points of intact proteins as identified by mass spectrometry. [d] Masses and isoelectric points of proteins (or their fragments) as identified by 2D electrophoresis. [e] Proteins identified in form of fragments.

impression, sera of 11 individuals allergic to hen's egg were pooled and used for immunodetection (**Figure 3**). As expected, the strongest binding of IgE was observed in process steps 1 and 2. Due to the intensity no differentiation between the single allergens in the M_r region between 28 and 70 kDa is possible. Although the antibody reaction becomes weaker from step to step, two discrete IgE binding bands emerge. These are recognized by the antisera also at the end of the process. Even in the hydrolyzed liquid egg of step 9 weak staining of these two bands is detected. In steps 3 and 4 many of the allergens are digested to smaller fragments. These, however, incorporate epitopes that were recognized by the pooled sera. At the end of the process in step 8 and especially in step 9, the allergenic potential is very low. This obviously parallels the protein breakdown shown in **Figure 2**.

Quantitation of the Immunoreactivity of Hen's Egg Proteins. IgE binding of the protein extracts obtained after the nine process steps was determined by the EAST inhibition test. The results are depicted in **Figure 4**, and the corresponding C_{50} values are shown in **Table 1**. The curves can be clearly divided into three groups: 1−4, 5–7, and 8 + 9. As observed for the immunoblot, this is in agreement with the protein pattern detected during 1D SDS-PAGE. Many proteins were observed in process steps 1–4, only a few were stained in lanes 5–7, and nearly none were seen in steps 8 and 9. This result is underscored by the C_{50} values. For the first four protein extracts the C_{50} values are in the low micrograms per milliliter range. Extracts from process steps 5–7 result in 260−403 μg/mL, and for steps 8 and 9 the inhibition at 10000 μg/mL was only 23%, indicating that nearly no immunoreactivity is left.

Protein Identification of Three Processing Steps by 2D Gel Electrophoresis and Mass Spectrometry. To elucidate changes of the molecular mass generated by the industrial process, the protein extracts obtained from steps 1, 3, and 9

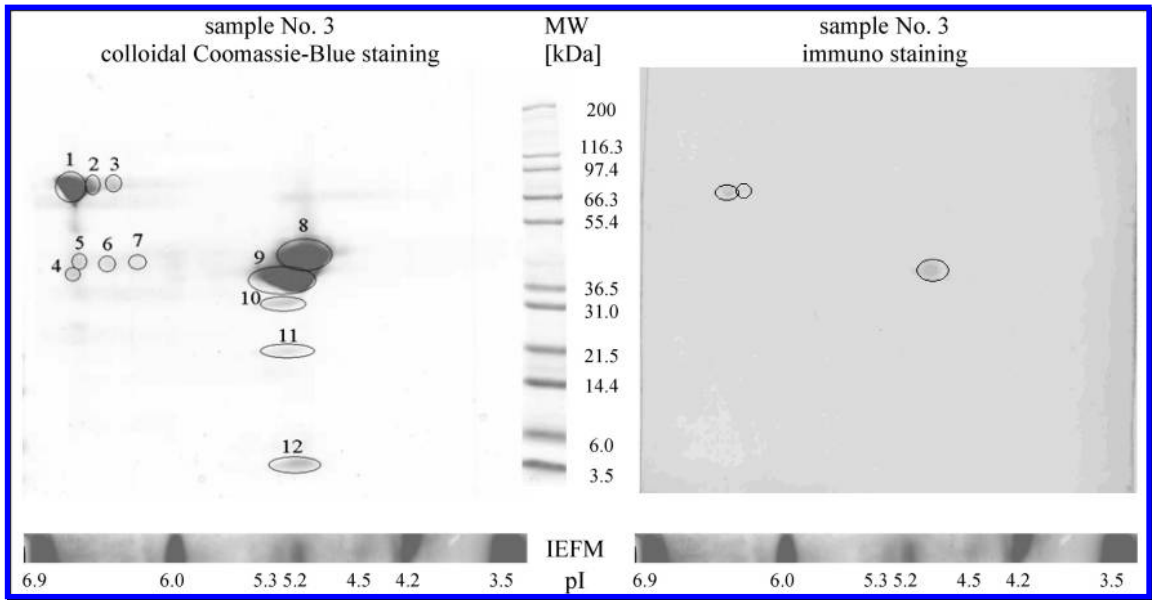

Figure 6. 2D electrophoresis of process step 3 with the corresponding immunoblot. Numbers indicate samples investigated by MALDI-MS (see **Table 3**).

Table 3. Mass Spectrometric Identification of Proteins from Process Step 3 Separated by 2D Gel Electrophoresis (See **Figure 5**)[a]

	Mascot data						2D electrophoresis			
no.	MS score	SC (%)	MS/MS score	peptide[b] score	mass[c] (kDa)	p*I*[c]	mass[d] (kDa)	p*I*[d]	IB	protein
1	305	56	245	100/145	77.5	7	~78	~6.7	+	conalbumin
2	183	41	65	29/36	77	7	~78	~6.7	+	conalbumin
3	142	61	169	74/95	78	7	~78	~6.7	−	conalbumin
4	161	32	122	53/69	79.6	8	44	~6.7	−	conalbumin
5	153	40	102	45/57	79.6	7	80	~6.7	−	conalbumin
6	116	51	207	91/116	43	5	44	~6.5	−	ovalbumin
7	115	61	206	84/122	43	5	43	~6.3	−	ovalbumin
8	165	62	183	118/66	43	5	45	~5.2	+	ovalbumin
9	135	52	243	119/34	43	5	40	~5.2	−	ovalbumin
10	103	39	237	116/121	43	5	45	~5.2	−	ovalbumin
11	99	47	265	78/84/102	43	5	21	~5.2	−	ovalbumin[e]
12			152	55/97	43	5	3.5	~5.2	−	ovalbumin[e]

[a] Score values for peptide mass fingerprint (MS score) and peptide fragment fingerprint (MS/MS score) as well as single peptide scores and sequence coverage (SC) are as determined by the Mascot algorithm. [b] Values for different peptides are separated by (/) and have a significance threshold of 41−44 (larger values represent identity). [c] Masses and isoelectric points of intact proteins as identified by mass spectrometry. [d] Masses and isoelectric points of proteins (or their fragments) as identified by 2D electrophoresis. [e] Proteins identified in form of fragments.

were separated by 2D gel electrophoresis. The Coomassie-stained spots were punched out and subjected to a fully automated protein identification with tryptic in-gel digest and MALDI-MS as described under Materials and Methods. The immunoreactivity was also tested by Western blotting from a second 2D gel, which was blotted to nitrocellulose and developed with the above-described pooled sera. Thereby, in addition to the molecular mass, charge differences generated during the technological process can be detected. Amounts of 59, 72, and 25 μg of protein extracts from steps 1, 3, and 9 were used. In **Figures 5** and **6** the Coomassie-stained gels together with the corresponding immunoblots are shown, and in **Tables 2** and **3** the mass spectrometric results are summarized. From process step 1 eighteen spots were subjected to mass spectrometric identification. One to three were unambiguously identified as conalbumin (ovotransferrin). However, the immunoreaction of these spots as also of spot 4 is very weak. A chicken protein, which is similar to ovoinhibitor (accession no. gi 71895337) is identified with a sequence coverage of 41% in the PMF and additionally by two high-scoring peptide sequences with MS/MS. The sera do not react with this molecule. Ovalbumin identified in spot 14 represents the highest quantity of all proteins. It is, however, not at all the strongest spot in the immunoblot. The predominant reaction is found in spots 15−17. These were identified as ovomucoid contaminated by very small amounts of ovalbumin. Spots 5, 6, 12, and 13 also contain ovalbumin. In spots 9–11 minor and major vitellogenin (VIT_1, accession no. gi 3123014; and VIT_2, accession no. gi 138595) were determined. From both proteins only peptides of the C-terminal regions were found. This is in agreement with the molecular mass of about 43 kDa. The protein detected obviously represents a C-terminal fragment of vitellogenin and not the complete protein. Spots 8 and 18 could not be identified by mass spectrometry.

Within process step 3 (**Figure 6** and **Table 3**) the degradation of the proteins proceeds. Only two proteins were identified: conalbumin (spots 1–5) and ovalbumin (spots 6–12). Thereby also the allergenic potential is reduced. Only three spots are recognized in the Western blot. The investigation of process step 9 demonstrates the nearly complete digestion of the egg proteins. There is extremely weak staining by Coomassie, and the sera do not react at all, indicating that no detectable allergenic potential is left (results not shown).

DISCUSSION

Hen's egg is used in many food formulations because it has excellent properties for the production of convenient products. Foaming, gelling, and emulsifying are desired properties in many industrial processes, and thus eggs are ingredients of many day-to-day foods. Even more important is the nutritive value of hen's egg. To make this available also for individuals who are allergic to hen's egg, special treatments are necessary. The influence of heat treatment and enzymatic digestion on the allergenicity of purified egg proteins has been described either alone or in combination (*5–8*). Necessary for the general use of whole eggs is the evaluation of the allergenicity of all egg constituents by as wide a range of sera of allergic people as possible.

The subject of these investigations has been a nine-step process consisting of heat and enzymatic treatments of whole eggs. The protein content and the IgE binding properties are characterized by immunostaining, mass spectrometric identification, and EAST inhibition. The single steps can be characterized only partly. Nevertheless, a few general features are obvious. The pasteurization performed to reduce the bacterial contaminations mainly influenced lysozyme. It is known to be a heat labile allergen (*18*), and hence it has been destroyed during the first step. No identification was possible. Stir heating for 10 min was presumably applied to further denature the proteins and render them more prone to the enzymatic digestion that followed in step 3. After 10 min of heating at 65 °C, no effect was observed. Due to the methods used it could not be differentiated between the degrees of denaturation. The protein compositions determined after 1 and 2 h of enzymatic digestion show no significant differences. The result of the SDS-PAGE is confirmed by the immune reaction. Neither increase nor decrease of IgE binding is observed in steps 3 and 4. As expected, however, the protein pattern and also the pattern of the Western blot indicate the efficacy of the enzyme treatment. Bands are shifted to smaller molecular mass fragments. The hydrolysis products representing fragments of the larger proteins still represent allergens that are recognized by the pooled sera and are therefore not convenient as ingredients to produce food for allergic individuals. Unexpectedly, the following heat treatment changed the protein composition considerably. It can be speculated that either the higher temperature led to the observed polypeptide cleavage or that the enzyme used in steps 3 and 4 for a short time becomes more active at the higher temperature before it is finally inactivated, rendering the solution ready for enzyme B used in steps 6 and 7. As observed in steps 3 and 4 for enzyme A, the digestion with enzyme B is finished after 1 h. Comparing steps 5 and 6, there is nearly no protein breakdown observed. The staining intensity varies only in a region where the glycoproteins ovomucoid and ovalbumin are observed. With respect to IgE binding, the broad bands characteristic of glycoproteins disappear and two sharp bands react with the pooled sera. As reported for step 5, the increase of temperature to 90 °C for 30 min in step 8 caused the nearly complete digestion of hen's egg proteins. Only very faint silver staining remains. The interpretation reported for step 5 may be also valid for step 8. Increase of temperature may induce stronger denaturation of the egg proteins, rendering them even more susceptible to digestion. Additionally, the enzyme may be more active at the higher temperature before it is inactivated. Step 8 has a very low immunoreactivity and is nearly identical to the end of the process shown in step 9. After careful consideration, there is no silver staining and no Coomassie staining of the two sharp bands recognized by the antisera. This implies that the epitopes recognized by the pooled sera do not represent proteins or the silver staining could not be made sensitive enough. No efforts were made to determine the nature of these bands.

These qualitative data are confirmed by the quantitative data measured in the EAST inhibition test shown in **Figure 4**. A reduction of the IgE binding potential was detected by EAST inhibition in samples 5−9. Due to the heat treatment in samples 5 and 8 the conformational structure of the allergens can be changed as a result of the loss of the tertiary structure (*24*). The heating to 75 °C for 10 min (sample 5) also showed a high reduction of the allergenic potential of >45-fold in comparison to sample 4 and >100-fold in comparison to sample 1. For process steps 8 and 9 only 23% EAST inhibition was determined.

Therefore, with the described process the aim was achieved to generate a product from hen's egg with very low allergenic potential. If eggs are pretreated according to the described procedure, allergenicity could be strongly reduced while texturizing and taste properties were preserved, from comparison of products such as rice cake or pudding made from either pasteurized egg (sample 1) or the product produced in step 9 of the process described here.

ACKNOWLEDGMENT

We thank Knut Brockow and Johannes Ring from the Technical University of Munich (Department of Dermatology and Allergology), Barbara Ballmer-Weber, Manjula Dutta, and Barbara Theler from the University Hospital of Zurich (Allergiestation, Dermatologische Klinik), Alessandro Fiocchi, Luigi Terracciano, and Teresita Sarratud from the Macedonio Melloni Hospital of Milan (Department of Pediatrics), and Radvan Urbanek, Zsolt Szepfalusi, Veronika Kirchlechner, and Eleonora Dehlink from the Medical University of Vienna (Department of Pediatrics and Juvenile Medicine) for providing the human sera. We give special thanks to Bettina Jacobsen for technical support.

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Received for review August 31, 2007. Revised manuscript received December 12, 2007. Accepted December 24, 2007. This research project was supported by the EU project REDALL (Reduced Allergenicity of Processed Food), QLK1-CT-2002-02687.

JF0725981