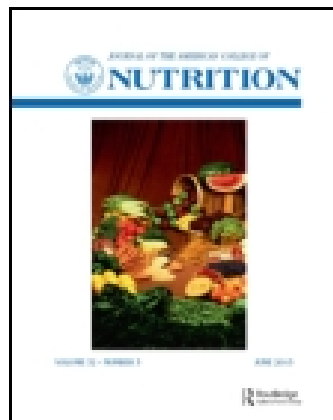




Journal of the American College of Nutrition

Publication details, including instructions for authors and subscription information:
<http://www.tandfonline.com/loi/uacn20>

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A Fiocchi^a, P Restani^a, E Riva^a, A R Restelli^a, G Biasucci^a, C L Galli^a & M Giovannini^a

^a 5th Pediatric Department, Institute of Pharmacological Sciences, University of Milan, Italy.

Published online: 04 Sep 2013.

To cite this article: A Fiocchi, P Restani, E Riva, A R Restelli, G Biasucci, C L Galli & M Giovannini (1995) Meat allergy: II-Effects of food processing and enzymatic digestion on the allergenicity of bovine and ovine meats., Journal of the American College of Nutrition, 14:3, 245-250, DOI: [10.1080/07315724.1995.10718503](https://doi.org/10.1080/07315724.1995.10718503)

To link to this article: <http://dx.doi.org/10.1080/07315724.1995.10718503>

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Meat Allergy: II - Effects of Food Processing and Enzymatic Digestion on the Allergenicity of Bovine and Ovine Meats

Alessandro Fiocchi, MD, Patrizia Restani, PhD, Enrica Riva, MD, Anna Rosa Restelli, PhD, Giacomo Biasucci, MD, Corrado L. Galli, PhD, and Marcello Giovannini, MD, FACN

5th Pediatric Department (A.F., E.R., G.B., M.G.), and Toxicology Laboratory (P.R., A.R.R., C.L.G.), Institute of Pharmacological Sciences, University of Milan, Italy

Key words: food allergy, bovine meat, ovine meat, serum albumins, technologically treated products, enzymatic digestion

Objectives: This study was designed to evaluate whether peptic treatment of BSA and OSA affects their allergenicity and to evaluate the effect of technological treatments of meat for infant feeding on the allergenicity of these proteins.

Subjects: Twelve children (8 males and 14 females) suffering from atopic dermatitis (AD), aged 12 months to 4.33 years (mean age 2.21 ± 1.05 years).

Study Design and Methods: Children suffering from atopic dermatitis (AD), clinically reactive and SPT-positive to beef, underwent skin prick testing (SPT) and radioallergosorbent test (RAST) with BSA and OSA, before and after peptic treatment. They were tested also with commercially available homogenized bovine meat (HBM) and with freeze-dried bovine (FDBM) and ovine (FDOM) meats. Peptic digestion of BSA and OSA was carried out in a Dubnoff's water bath containing 0.05 mg/ml of pepsin. SPT and RAST were performed for 1 mg/ml of BSA and OSA digested 5 minutes, 2 hours and 4 hours with pepsin; homogenized bovine meat, and FDBM and FDOM. SPT results were expressed as diameters (in mm) of the wheal obtained after SPT; a diameter ≥ 3 mm was considered as positive. RAST was considered positive for IgE values $5 > \text{UI/ml}$.

Results: SPT to native BSA and OSA were positive in all patients. Only 2 of the 12 children were SPT-positive to HBM, FDBM, and FDOM. After digestion, SPTs were positive as follows: for BSA, 4/12 after 5 minutes peptic treatment, 2/12 after 2 hours and 2/12 after 4 hours; for OSA, 3/12 after 5 minutes peptic treatment, none after 2 and 4 hours. None of RASTs was positive after peptic attack.

Conclusions: Both proteolytic digestion and technological treatment reduced the allergenic potential of meat products.

INTRODUCTION

In a previous paper [1], we reported data supporting the role of bovine serum albumin (BSA) and ovine serum albumin (OSA) in the allergenicity of beef and lamb, respectively. A cross-reactivity between the two serum albumins was also suggested.

In a double-blind placebo controlled food challenge (DB-PCFC), the detection of specific IgE for BSA and OSA by skin prick test (SPT) and radioallergosorbent test (RAST), in children suffering from atopic dermatitis (AD), was not always associated with clinically relevant symptoms [1]. The lack of

correlation between SPT and food challenge responses has also been reported by other authors [2].

If serum albumins are the most important meat allergens, their denaturation by heating (cooking) and/or enzymatic proteolysis (digestion) could explain why many children, showing specific IgE, are clinically tolerant to meat. To date, little is known about the effects of food processing (i.e., heating, homogenization and freeze-drying) or enzymatic attack on meat allergy. However, clinical tolerance to the ingestion of cooked fish has been previously described in subjects with immunological evidence of antibodies for antigen M, which is present in all types of fish [3,4].

Address request reprints to: Alessandro Fiocchi, MD, 5th Pediatric Department, Ospedale S. Paolo, via Di Rudinì, 8, 20142 Milano, Italy.

On the basis of the hypothesis that the technological processes and/or the enzymatic digestion could modulate the allergenicity of meat proteins, we report data on the SPT- and RAST-responses to technologically-treated meat samples, and to BSA or OSA before and after enzymatic attack. Since lamb is often proposed as a substitute for beef and milk substitute [5,6] in atopic children, we investigated whether a different pattern of serum albumin gastrointestinal proteolysis could justify the different allergenic potential of bovine and ovine derivatives.

CLINICAL DATA AND METHODS

The selection of bovine meat-SPT-positive children was performed during the period October 1, 1991 to the February 29, 1992, starting from a group of 134 children suffering from AD. Of these, 38 (21 males and 17 females) were SPT-positive to cow's milk. Their ages ranged from 9 months to 8 years (average 2.97 ± 2.76). The 12 atopic children considered in this study are those presented in a previous paper [1], where clinical characteristics are also reported. There were 8 males and 4 females and their ages ranged from 1 to 4.33 years (average 2.21 ± 1.05). All children were also allergic to cow's milk.

In vitro Digestion of Serum Albumins

Samples of serum albumins (BSA and OSA) were weighed into 20-ml screw-cap flasks and suspended in 0.01 N HCl, pH = 2, containing 0.05 mg/ml of pepsin [7]. The final protein concentration was 3.0 ± 0.1 mg/ml and the enzyme/protein ratio was 1/60 (w/w). The protein content of purchased BSA and OSA was verified by Lowry's method [8].

Proteolysis was carried out at 37°C in a Dubnoff's water bath shaken at 100 beats/min. The reaction was stopped at different times during pepsin attack (5 minutes, 2 hours and 4 hours) by rapid freezing in acetone and dry ice. Samples were then stored at -80°C and freeze-dried within a few days.

SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a gradient running gel (9–19% acrylamide, pH = 8.8) that contained 0.1% of SDS [7].

Meat samples (raw, homogenized and freeze-dried bovine products; raw and freeze-dried ovine products) were suspended in sample dilutor (0.25M Tris-HCl buffer, pH = 6.8, containing 7.5% glycerol, 2% SDS and 1% b-mercaptoethanol) at the concentration of 3.0 mg/ml and then heated for 10 minutes at 100°C.

BSA and OSA (before and after pepsin proteolysis) were diluted in sample dilutor (without b-mercaptoethanol; 1 mg/ml) and treated as described above. Molecular weight marker

solution included: rabbit skeletal muscle myosin (MW = 200000), E. Coli b-galactosidase (MW = 116250), rabbit muscle phosphorylase b (MW = 97400), bovine serum albumin (MW = 66200), hen egg white ovalbumin (MW = 42700), bovine carbonic anhydrase (31000), soybean trypsin inhibitor (MW = 21500), hen egg white lysozyme (MW = 14400), bovine pancreas aprotin (MW = 6500). After loading the samples (20 ml each) into the gel, the run was performed at 100 mV for 4 hours. Gels were dyed with Coomassie Brilliant Blue G-250 according to Neuhoﬀ et al [9]. All chemicals used in electrophoretic analyses were from BioRad (Richmond, CA).

SPT

During April 1992, the children underwent SPT according to Bock [10]. The following allergens were tested: BSA and OSA (Sigma) at the highest available purification level [1]; BSA and OSA digested 5 minutes, 2 hours, and 4 hours with pepsin commercial homogenized (strained) bovine meat and commercial freeze-dried bovine and ovine meat.

BSA and OSA samples were solubilized in glycerol 50% (in water) at the initial concentration of 1 mg/ml and then diluted several times (1:10 v:v) until the endpoint was reached. The homogenized sample was suspended 1:10 (v:v) in 50% glycerol. Freeze-dried meat preparations were suspended in 50% glycerol at the final concentration of 20 mg/ml.

SPT for raw bovine and ovine meats (prick by prick) was performed according to Ortolani [11].

Results were expressed as diameters (in mm) of the wheal obtained after SPT; a diameter of 3 mm was considered positive [12].

RAST

On the same day, a venous blood sample was taken from each child to evaluate the total content of IgE and to perform the RAST for the allergens already assayed in SPT. RAST was performed by Bayer Laboratories (Garbagnate, Milano-Italy) using disks obtained according the following protocol: samples of 0.5 to 2 mg (BSA and OSA, before or after peptic digestion, homogenized and freeze-dried meats) were solubilized in 1 ml of phosphate buffer (PBS), gently shaken for 4 hours, centrifuged and filtered on Millex 0.8 and 0.22 mm (Millipore, Bedford, MA, USA). Samples were then mixed with paper disks activated by cyanogen bromide (Pharmacia, Uppsala) to allow the binding of the allergen to the disk. Specific IgE values higher than 5 UI/ml were considered RAST-positive [13].

Statistical Analyses

Statistical analyses were performed according to Chi-square test and to Spearman's rank test.

RESULTS

SDS-PAGE

Meat Samples. Figure 1 shows the electrophoretic behavior of bovine and ovine meat samples. The protein distribution is similar in the two raw meats, confirming the phylogenetic relationship of the two animal species. One of the most important differences observed in these protein patterns is the higher quantity of serum albumins present in raw meats, while the corresponding band in technologically treated samples is less visible. The reduction in serum albumin content is more evident in ovine freeze-dried meat than in bovine treated products.

Digested Serum Albumins. Figure 2 illustrates the electrophoretic patterns of BSA and OSA before (0 minutes) and at different times during peptic attack (5 minutes, 2 and 4 hours). Pepsin attack on the two serum albumins is different as shown by the number and the quality of peptide fragments produced. After 5 minutes of digestion, BSA presents a higher number of proteic bands than OSA. Moreover, in OSA there is a rapid reduction of high-molecular-weight fragments and a higher release of low-molecular-weight polypeptides.

The difference between the two proteins is reduced after 2 hours and 4 hours of digestion, where polypeptidic fragments are similar in molecular weight but different in relative abundance.

SPT for BSA and OSA Before and After Peptic Digestion

All the children included in this study were SPT-positive for BSA and OSA. The specific endpoints for BSA and OSA were reached respectively: at 1 mg/ml in 2 and 5 children; at 0.1 mg/ml in 6 and 3 children; at 0.01 mg/ml in 2 and 4 children; and at 0.001 mg/ml in 2 BSA-positive children. A significant correlation (Spearman's test) between wheal diameters ob-

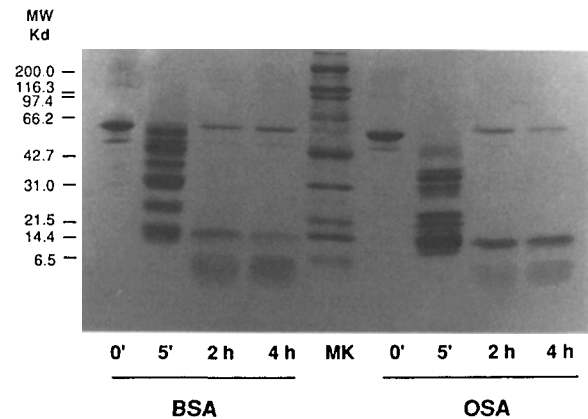


Fig. 2. SDS-PAGE of BSA and OSA before (0 minutes) and after different times (5 minutes, 2 hours and 4 hours) of peptic digestion. MK = MW marker solution.

tained in SPT for the two native proteins was found ($r = 0.83$; $p < 0.001$).

After 5 minutes of peptic attack, only four children maintained their SPT-positivity to BSA and three children were positive to OSA (at the concentration of 1 mg/ml) (Table 1). The endpoints for the two albumins were reached as follows: BSA: 3 out of 4 children at 1 mg/ml and the fourth at 0.01 mg/ml; and OSA: 2 out of 3 children at 1 mg/ml and the third at 0.1 mg/ml.

After 5 minutes of digestion, the reduction of SPT-response both to BSA (chi-square = 9.187; $p = 0.002$) and to OSA (Chi-square = 11.378; $p < 0.0001$) was statistically significant when compared to the native protein (0 minutes). Moreover, Spearman's test showed a significant correlation between wheal diameters, obtained with the two albumins digested for 5 minutes ($r = 0.69$ and $p = 0.013$). The decreasing of SPT-positivity became more evident after longer enzymatic attack. In fact, after 2 hours of digestion, only two children reacted to

Table 1. SPT for Bovine and Ovine Serum Albumins Before and After Peptic Digestion (Positive Responses/Total)

Concentrations (mg/ml)		BSA	OSA
0 minute PS	1	12/12	12/12
	0.1	10/12	7/12
	0.01	4/12	4/12
	0.001	2/12	0/12
	0.0001	0/12	0/12
5 minutes PS	1	4/12	3/12
	0.1	1/12	1/12
	0.01	1/12	0/12
	0.001	0/12	0/12
2 hours PS	1	2/12	0/12
	0.1	0/12	0/12
	0.01	0/12	0/12
4 hours PS	1	2/12	0/12
	0.1	0/12	0/12
	0.01	0/12	0/12

PS = pepsin attack

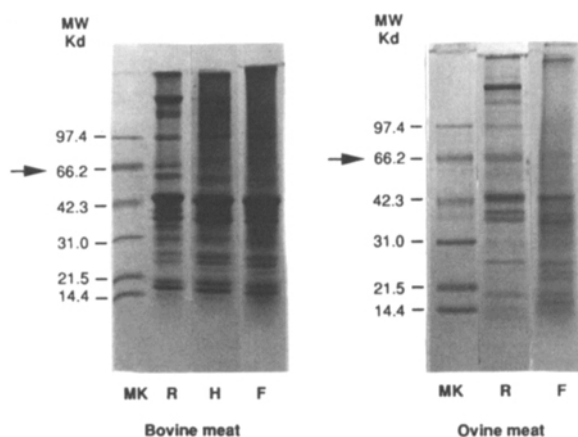


Fig. 1. SDS-PAGE of bovine and ovine meat products. MK = MW marker solution; R = raw meat; H = homogenized meat; F = freeze-dried meat. Arrows indicate the position of serum albumins.

BSA (1 mg/ml) and none to OSA. All children who were SPT-positive to BSA presented the endpoint at the concentration of 1 mg/ml. At lower concentrations no child showed a wheal diameter > 3 mm. After 4 hours of peptic digestion, there was no further reduction of SPT-positivity; in fact, the two children positive to BSA after 2 hours of digestion were still reactive at this time.

SPT for Processed Meat Products

Spearman's test showed a significant correlation ($r = 0.60$ and $p = 0.039$) between wheal diameters obtained by SPT with the raw bovine and ovine meats.

Only 1 of the 11 children STP-positive to raw bovine meat and 1 of the 12 children STP-positive to raw ovine meat responded to homogenized and freeze-dried products (Table 2). When evaluated with the Chi-square test, the SPT responses had the following statistical significance: the comparisons between raw bovine meat and both homogenized and freeze-dried bovine meat had Chi-square = 13900, $p < 0.0001$; the comparison between raw ovine meat and the corresponding freeze-dried product had Chi-square = 16783, $p < 0.0001$.

RAST for Bovine and Ovine Products

Compared to SPT, RAST presented a different pattern of positivity in the 12 children considered in this study. Only 5 of 12 (41.7%) SPT-positive children were RAST-positive for BSA, and 3 (25%) for OSA (Table 3). The RAST-positivity for both BSA and OSA was lost after only 5' of peptic digestion.

No RAST-positivity was observed with homogenized bovine meat, while one child responded slightly to both bovine and ovine freeze-dried preparations (Table 4).

DISCUSSION

Though not necessarily associated with clinical intolerance, the presence of specific IgE for meat products often results in the exclusion of these important protein sources from the diet of children with AD. This has very serious nutritional implications since children allergic to beef are generally also reactive to cow's milk, another important protein source. The use of

Table 3. RAST Responses to BSA and OSA Before and After Peptic Digestion

Product tested	Positive responses (n = 12)
Bovine serum albumin	
2 mg/ml	5
1 mg/ml	4
0.5 mg/ml	4
5 minute PS	0
2 hour PS	0
4 hour PS	1
Ovine serum albumin	
2 mg/ml	3
1 mg/ml	2
0.5 mg/ml	2
5 minute PS	0
2 hours PS	0
4 hours PS	0

PS = Peptic attack

Table 4. RAST Responses to Bovine and Ovine Treated Meats

Product tested	Positive responses (n = 12)
Homogenized bovine meat	
1 mg/ml	0
2 mg/ml	0
1:10 mg/ml	0
Freeze-dried bovine meat	
1 mg/ml	1
2 mg/ml	1
1:10 mg/ml	1
Freeze-dried ovine meat	
1 mg/ml	1
2 mg/ml	1
1:10 mg/ml	1

lamb meat as an alternative for children allergic to bovine derivative could be questionable, since, as we showed previously, a cross-reactivity exists between the two animal species, at least in SPT [1]. We also showed that serum albumin is an important, if not the main meat allergen.

The chemical structure of bovine [14] and ovine [15] serum albumins are well known, as are their main physico-chemical characteristics [16]. Serum proteins are not heat-resistant: their structure is affected by heating at 60°C and their allergenicity is lost at higher temperatures [17]. Technological processes are able to change some properties of meat proteins, with a modification of their electrophoretic behaviour (Fig. 1) and a reduction of their digestibility [1]. These effects are mainly due to the heating (steam injection at 110–120°C) performed in the food industry as the first step in the production chain of homogenized and freeze-dried meat products. Serum albumins are clearly evident in raw meats, while their intensity is lower

Table 2. SPT for Raw and Processed Meat Products

Product	Positive responses (n = 12)
<i>Bovine products</i>	
Raw meat	11
Homogenized meat	1
Freeze-dried meat	1
<i>Ovine products</i>	
Raw meat	12
Homogenized ovine meat	1
Freeze-dried ovine meat	1

in the technologically treated samples. This consideration correlates well with the observed lower SPT-positivity of homogenized and freeze-dried products when compared to the corresponding raw meats. On the basis of these results, we can further support the hypothesis of the involvement of serum albumins in meat allergy. The lower quantity of serum albumin observed in ovine freeze-dried, compared to the corresponding bovine product, seems to indicate a higher heat-lability of OSA, with a decreased allergenic potential of ovine treated meat. Thus, the lower allergenicity of ovine meat, observed in clinical practice, could be partially supported by the stronger thermodenaturation of the serum albumin.

The peptic digestion of serum albumin decreases the allergic potential of both proteins, even after only 5 minutes of enzymatic action. At this time of proteolysis, 4 of the 12 children included in the study, reacted to BSA and three reacted to OSA. This fact correlates well with the electrophoretic behavior observed in Fig. 2 where the release of peptidic fragments with low MWs is evident. The protein fragments are less allergenic than native protein [18]. The phenomenon is more evident in the case of OSA, after 5 minutes of digestion it releases fragments with MWs lower than those of BSA which could explain the different responses of allergic children. The allergic properties of the two albumins decrease further when a longer time of digestion is considered. After 2 hours, only two children react to BSA and none to OSA. Although similar in MWs, the different amino acid sequence of the polypeptidic fragments still present in the digestion mixture coming from BSA and OSA (Fig. 2) could be responsible for the clinical result. After 4 hours of digestion, BSA still produces an SPT-positive response in two children, the same subjects who were positive at 2 hours. Probably this result was due to the inability of pepsin to hydrolyze BSA further and to eliminate the peptidic fragments still allergenic for the most sensitive children. This situation further supports the previous hypothesis that the limited differences seen in the two amino acid sequences (55 out of 607 amino acids) could play an important role in modulating the albumin antigenicity. The rapid disappearance of SPT-reactivity for OSA as a consequence of the peptic digestion further justifies the observed lower clinical allergenicity of ovine meats in comparison to bovine products.

The RAST test always shows a lower positivity in comparison to SPT. For example only 5 of the 11 SPT-positive children for BSA are RAST positive and only 3 of 12 are RAST-positive for OSA. When digested albumins are tested, no positivity is evident after 5 minutes of peptic digestion. This could be attributed to a lower sensitivity of RAST in comparison to SPT, or to the well known phenomenon of tolerance that makes subjects who are SPT-reactive for some food allergens clinically asymptomatic [19]. The results of the double-blind oral challenge test, described elsewhere [1], showed few children positive to BSA (2/12) and to OSA (5/12), further supporting the hypothesis of tolerance.

In conclusion, our results indicate that technological pro-

cesses and proteolysis drastically reduce the allergenicity of meat products, so that the elimination of these important protein sources from the diet of atopic children should be reconsidered. The lower allergenicity of ovine meat, in comparison with the corresponding bovine products, seems due to the higher thermosensitivity and to the faster proteolysis of OSA. Thus, the clinical practice suggesting ovine products as an alternative diet for polyallergic children could be biologically justified but only in selected cases. On the other hand, the use of specific technological processes could permit the introduction of bovine meat in the diet of the children allergic to milk without risk of negative reactions.

ACKNOWLEDGMENTS

We thank Mrs. Helen Downes for editorial assistance.

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Received February 1994; revision accepted October 1994.