

# Antigenic Determinants of Bovine Serum Albumin

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## Key Words

Bovine serum albumin · Food allergy · Epitopes ·  
Immunochemistry

## Abstract

**Background:** Bovine serum albumin (BSA) is one of the most widely studied proteins; its structure is well known and its antigenic characteristics have been described in several papers. The aim of this research was the identification of the BSA antigenic determinants. **Methods:** This study was performed using limited proteolysis and an immunoblotting technique, in which a commercial murine antibody and sera from children sensitized to BSA were used. **Results:** Findings suggest amino acids (aa) 524–598 as an epitopic area for human species. The most critical sequence seems to be aa 524–542, even if it must be included in a longer fragment to be recognized by antibodies. Murine IgG antibodies also recognize fragments contained in the first half (NH<sub>2</sub>-terminal portion) of BSA. **Conclusions:** The results presented in this study indicate that the epitopic sites of an antigenic protein can be different when different species are considered, so that data obtained with antibodies from animal species cannot be directly extrapolated to the behavior of human IgEs.

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## Introduction

The antigenic properties of bovine serum albumin (BSA) have been reported by numerous authors. BSA is involved in milk allergy, where it represents one of the most important antigens from whey proteins [1–4]. The importance of BSA as an allergenic molecule in patients with cow's milk allergy has been demonstrated by oral challenge [1], skin prick test [2, 3] and 'in vitro' assays [3, 4]. Patients allergic to animal's hair/dander normally react to the serum albumin of the correlated species; subjects allergic to cows often react to BSA [4, 5]. In our previous studies, BSA was also identified as one of the most important allergens in bovine meat as shown by skin prick test, immunoblotting and double-blind placebo-controlled food challenge (DBPCFC) [6–8].

BSA is one of the most widely studied proteins; its complete amino acid sequence has been determined [9] and models of its three-dimensional conformation have been developed [10, 11]. The primary structure of BSA is organized into three homologous repeated units or domains (1–190, 191–382, 383–581) and consists of nine separate disulfide-bonded loops connected by peptide links of 11–26 residues [11–13].

The presence of disulfide bonds in BSA has been indicated as an important factor in maintaining the native antigenic determinants of this molecule; Habeeb and Borella [14] showed that the reduction of disulfide bonds,

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which is associated with important conformational changes, completely abolishes the reaction of BSA with anti-BSA antibodies. In contrast, previous studies by our group showed that serum albumin antigenicity is only partially correlated with its native three-dimensional structure [8].

Several studies have evaluated the antigenic potential of fragments deriving from limited proteolysis of BSA; in these assays, the formation of an immune complex was evaluated using sera from immunized animals. The epitopes identified in the various studies were not always in agreement and this fact could be correlated with the different experimental conditions. Using rabbit anti-BSA antibodies, Peters et al. [13] suggested that antigenic sites are distributed along the entire protein chain, with the sites of greatest antibody affinity situated in the HOOC-terminal region. Moreover, they presented evidence that some sites are homologous, reacting with the same populations of antibodies, while other sites bind an exclusive population of antibodies. Atassi et al. [15] and Habeeb and Atassi [16] identified two amino acid sequences as possible BSA epitopes: the tryptic fragments Phe 11–Arg193 and His377–Lys571. Each fragment inhibited the reaction of BSA with anti-BSA by 90% or better. Ferguson et al. [17] evaluated the immunogenicity of seven immunogenic BSA fragments obtained by incubating the protein with pepsin, trypsin or CNBr. In comparison to native BSA, some of them were only weakly immunogenic (CNBr505–546 and P505–582), while others (T377–582, T115–184 and P307–582) were highly immunogenic. Wahn et al. [18] showed that large fragments (P1–306, P307–582 and T377–582) could block BSA-antibody binding in rabbit antisera and induce histamine release from the basophils of both immunized rabbits and BSA-sensitive subjects. Finally, the residues 126–144 of BSA (ABBOS epitope) have been reported to be possibly responsible for an autoimmune reaction directed against pancreatic islet cells in insulin-dependent diabetes mellitus [19].

We showed in previous studies that circulating IgEs specific for BSA (from sera of allergic children) were able to cross-react with albumins from sheep and pig, but they did not recognize those of horse, rabbit, turkey and chicken [8]. We hypothesized that the BSA antigenic determinant for human antibodies is localized in the sequence 520–542, where important differences in the amino acid sequence are identifiable between cross-reacting and non-cross-reacting serum albumins.

The aim of the research presented in this paper was the confirmation of the amino acid sequence of the epitopic

site suggested in our previous paper; to reach this goal, we used limited tryptic proteolysis on BSA and anti-BSA antibodies from both immunized animals and sensitized subjects.

## Materials and Methods

### *Clinical Data*

Serum samples were taken from 4 children (3 males and 1 female). Their age at the time of serum collection ranged from 4 to 5.58 years (average  $\pm$  SD  $4.71 \pm 0.70$  years). The children included in this study reported a history of clinical reactions to beef; they showed a skin prick test-positive response to diagnostic reagents (Bayer, Garbagnate) for cow's milk and beef and to purified BSA (1 mg/ml in 50% glycerol; Sigma Aldrich, Milan, Italy). In the DBPCFC, performed as described by Fiocchi et al. [6], all children presented instantaneous reactions to purified BSA (Sigma Aldrich) with lip edema, rhinitis, urticaria and vomiting.

As a negative control in immunoblotting, sera from 10 nonallergic adults and from 10 children allergic to foods other than meat (and negative to BSA) were used.

Informed consent was obtained from the parents of all children participating in this study.

### *BSA Proteolysis*

BSA was purchased from Sigma Aldrich at the highest available purification level, i.e. an essentially globulin-free, lyophilized powder, approximately 99% by agarose electrophoresis. Trypsin (Sigma Aldrich) was from bovine pancreas, dialyzed and lyophilized, and essentially salt free; its activity was 10,000–13,000 BAEE units per mg of protein. BAEE = *N*-benzoyl-L-arginine ethyl ester (a trypsin substrate).

BSA was solubilized at the final concentration of 3 mg/ml in 0.05 M tris-(hydroxymethyl)aminomethane (Tris)-HCl buffer, pH 8, containing 0.025 mg/ml trypsin (enzyme to protein ratio 1:120, w:w). Proteolysis was carried out at 37 °C in a Dubnoff water bath shaken at 100 beats/min.

The reaction was stopped at different times (1, 5, 10 and 20 min and 1 h) by diluting each sample with the same volume of sample buffer [final concentration: 0.125 M Tris-HCl buffer, pH 6.8, containing 9.4% glycerol, 1% sodium dodecyl sulfate (SDS), 2.5%  $\beta$ -mercaptoethanol] and then heating for 10 min at 100 °C.

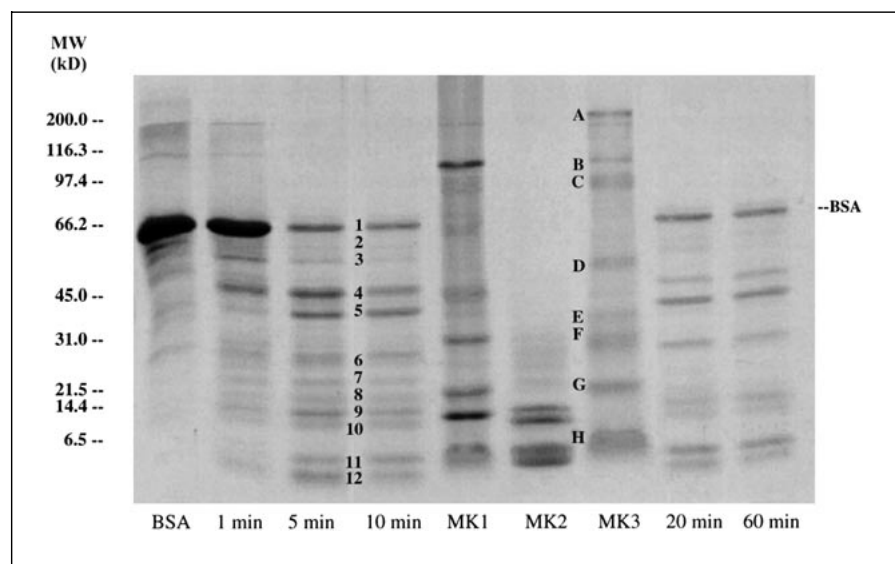
### *SDS Polyacrylamide Gel Electrophoresis*

All chemicals and instruments used in the electrophoretic analyses were purchased from Bio-Rad (Richmond, Calif., USA).

Samples were separated with a polyacrylamide gradient running gel (9–19%) according to Restani et al. [3].

After the electrophoretic run (90 V at room temperature, for approximately 6 h), the gels were dyed with Coomassie brilliant blue G-250 according to the method of Neuhoﬀ et al. [20]. Molecular weights (MWs) of tryptic fragments were calculated using two marker solutions. Marker 1 (MK1; broad range, BioRad) contained myosin (rabbit muscle; 200 kD),  $\beta$ -galactosidase (*Escherichia coli*; 116.3 kD), phosphorylase B (rabbit muscle; 97.4 kD), BSA (66.2 kD), ovalbumin (chicken egg; 45 kD), carbonic anhydrase (bovine erythrocytes; 31 kD), soybean trypsin inhibitor (soybean; 21.5 kD), lysozyme (chicken egg white; 14.4 kD) and aprotinin (bovine pancreas; 6.5 kD). Marker 2 (MK2; Sigma Aldrich) contained myoglobin

**Fig. 1.** SDS-PAGE of polypeptides obtained by limited tryptic digestion of BSA. Band 1 corresponds to native BSA; bands 2–12 are the main proteolytic fragments. MK1, MK2 and MK3 are the MW marker solutions; their composition is described in Materials and Methods. The MWs reported on the left of the gel correspond to the proteins contained in MK1. Capital letters are used to identify the correspondence between bands in the gel and in immunoblotting (prestained marker = MK3).



(polypeptide backbone 1–153; 16.95 kD), myoglobin I+II (1–131; 14.44 kD), myoglobin I+III (56–153; 10.6 kD), myoglobin I (56–131; 8.16 kD), myoglobin II (1–55; 6.21 kD), glucagon (3.48 kD) and myoglobin III (132–153; 2.51 kD).

To compare the bands on gels and membranes, we used a pre-stained broad-range MW marker (MK3; Bio-Rad) containing myosin (rabbit muscle; 203 kD),  $\beta$ -galactosidase (*E. coli*; 118 kD), BSA (86 kD), ovalbumin (chicken egg; 51.6 kD), carbonic anhydrase (bovine erythrocytes; 34.1 kD), soybean trypsin inhibitor (soybean; 29 kD), lysozyme (chicken egg white; 19.2 kD) and aprotinin (bovine pancreas; 7.5 kD).

The MWs of fragments were calculated using a gel scanner (Sharp JX-330, Pharmacia Biotech, Sweden) and the Image Master™ 1D Software.

#### Immunoblotting

After polyacrylamide gel electrophoresis (PAGE), immunoblotting was performed as described by Restani et al. [8].

The membranes were incubated overnight in 10 ml of 0.25% gelatin solution, containing 0.3 ml of serum from allergic children or 10  $\mu$ l of murine antibody (Sigma Aldrich).

Antigen-IgE complex (human serum) was detected using goat anti-human IgE antibodies (Sigma Aldrich), while antigen-IgG complex (murine antibody) was detected using goat anti-mouse IgG antibodies (Sigma Aldrich), both labeled with alkaline phosphatase; the secondary antibody commercial stock was diluted 1/1,000 (v:v) in 0.25% gelatin solution.

After incubation for 4 h at room temperature with shaking, membranes were washed twice with 0.25% gelatin solution (2 min each) and once with Tris buffer solution (20 mM Tris and 0.5 M NaCl) for 5 min.

Finally, after incubation in bromochloroindolyl phosphate-nitro-blue tetrazolium (BCIP-NBT) solution, an intense black-purple precipitate developed at the site of enzyme binding. The developing solution contained 15% BCIP and 30% NBT in alkaline phosphatase buffer (100 mM Tris, 100 mM NaCl, 5 mM  $MgCl_2$ , pH 9.5). Unless

otherwise specified, chemicals were obtained from Merck (Darmstadt, Germany).

#### Automatic Determination of the Amino Acid Sequence

Membranes prepared for sequencing were stained directly (0.1% Coomassie brilliant blue G-250 in 50% methanol and 10% acetic acid) and the bands were cut using a surgery scalpel.

Amino acid sequences were determined using a Perkin-Elmer Applied Biosystems 492 pulse liquid-phase sequencer, PTH-derivative amino acids were identified by RP-HPLC (Perkin-Elmer Applied Biosystems). PTH = Phenylthiohydrazide.

## Results

The electrophoretic distribution of BSA fragments deriving from limited tryptic proteolysis is shown in figure 1. Eleven polypeptides were liberated by enzymatic attack (bands 2–12). Some of them were released immediately (1 min, bands 2–4), while others appeared clearly only after 5 min (bands 5–12). After this time, the fragments with higher MW (bands 2–5) decreased or disappeared, while those having lower MW (bands 7–12) increased. The pattern remained qualitatively unmodified starting from 20 min of proteolytic attack.

Circulating IgEs from the four children included in this study were tested for reactivity against the proteolytic fragments from the attack of trypsin on BSA (fig. 2). The use of pre-stained MW standard solution (broad range) permitted correlation of the bands stained by Coomassie blue in SDS-PAGE with those identifiable in the immunoblotting membrane after incubation with the alkaline

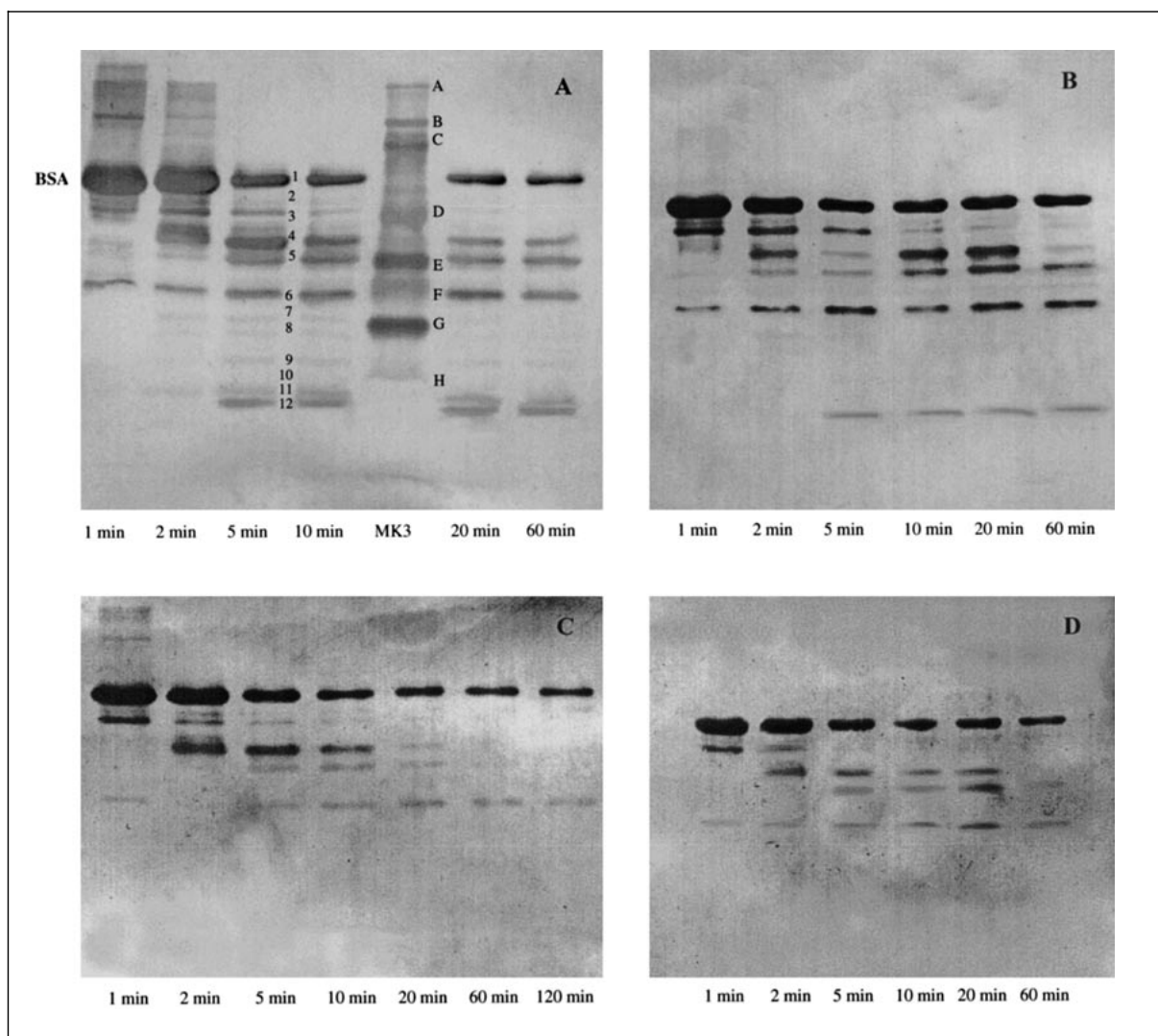


**Fig. 2.** Positive responses obtained by incubating a membrane with the same electrophoretic pattern as shown in figure 1 with the serum of child 1 (**A**), child 2 (**B**), child 3 (**C**) and child 4 (**D**). Capital letters in **A** are used to identify the correspondence with bands in the gel (fig. 1).

phosphatase substrate. Table 1 summarizes the positive responses to tryptic fragments of BSA obtained by using sera of the four children allergic to BSA. All children had circulating IgEs recognizing BSA and 4 out of 11 derived fragments, i.e. bands 3–6. Only two subjects (children 1 and 2) presented positive responses to the bands 11 and 12. No significant positive response was shown to bands 2 and 7–10. No significant positive response could be detected when nonspecific binding was investigated (serum from children allergic to foods other than milk and nonallergic adults).

In figure 3, immunoblotting obtained by incubating a membrane identical to that of figure 1 with a commercial anti-BSA antibody is shown. In this case, the antibody was able to recognize all the fragments present in the electrophoretic pattern.

In order to identify the epitopic area of BSA, we sequenced most of the BSA tryptic fragments. The results of the automatic determination of 8 amino acid sequences (bands 3–6 and 9–12) are illustrated in figure 4. Band 3 was only partially sequenced [from amino acid (aa) 460 to 607] because the NH<sub>2</sub>-terminal region was blocked, while band 7 was a complex mixture of different polypeptides.

**Fig. 3.** Positive responses obtained by incubating a membrane with the same electrophoretic pattern as shown in figure 1 with a commercial murine anti-BSA antibody. Capital letters are used to identify the correspondence between bands in the gel and in immunoblotting.

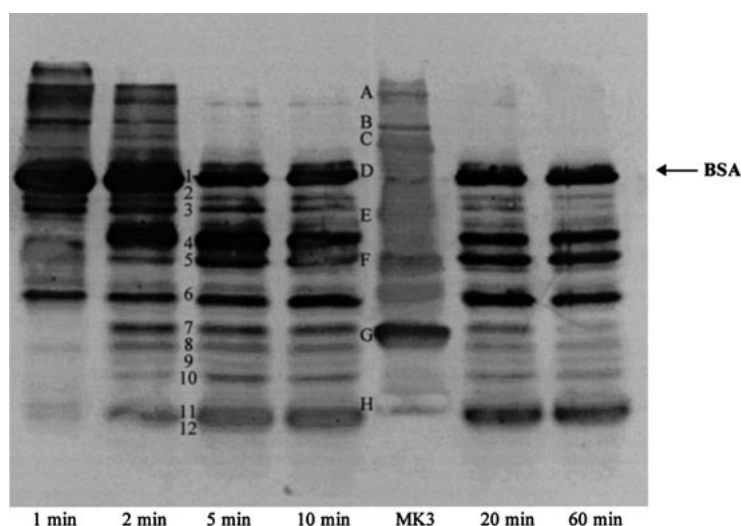


**Table 1.** Binding between BSA tryptic fragments and circulating IgEs from the 4 children included in the study

| Proteolytic fragment | IgEs    |         |         |         |
|----------------------|---------|---------|---------|---------|
|                      | child 1 | child 2 | child 3 | child 4 |
| BSA                  | +       | +       | +       | +       |
| Band 2               | –       | –       | –       | –       |
| Band 3               | +       | +       | +       | +       |
| Band 4               | +       | +       | +       | +       |
| Band 5               | +       | +       | +       | +       |
| Band 6               | +       | +       | +       | +       |
| Band 7               | –       | –       | –       | –       |
| Band 8               | –       | –       | –       | –       |
| Band 9               | –       | –       | –       | –       |
| Band 10              | –       | –       | –       | –       |
| Band 11              | +       | +       | –       | –       |
| Band 12              | +       | +       | –       | –       |

+ = Positive response; – = negative response.

Bands 2 and 8 were not sequenced because of their very short persistence and very low abundance, respectively.

All bands recognized by the human IgEs were at the HOOC-terminal region (bands 3–6 and 11), while other fragments (bands 8–10) did not bind the same antibodies. Three fragments co-ran in band 12; two of them were at the HOOC-terminal region of BSA (sequences 469–523

and 523–568) and one (sequence 139–183) was in the first part of the sequence.

Murine IgG antibodies presented a different behavior; in fact, they also recognized fragments contained in the first half (NH<sub>2</sub>-terminal portion) of BSA (bands 9 and 10).

## Discussion

Serum albumins are important proteins not only due to their presence in blood, where they act as carriers of different substances, but also their presence in some foods. BSA has been reported as one of the major allergens of milk [1–3] and meat [6–8].

The children included in this study presented immediate clinical reactions when BSA was administered in a DBPCFC [6, 7]. Before performing the oral challenge, serum was drawn from all children in order to continue our biochemical studies using an in vitro test (immunoblotting).

This technique is useful not only to improve our knowledge of the chemistry of this allergenic protein (epitope identification), but also to better evaluate its role in food allergy.

The evaluation of the immunochemical reactions obtained in immunoblotting showed that the BSA epitopic area can change when human and murine antibodies were

|     |             |            |            |            |            |            |
|-----|-------------|------------|------------|------------|------------|------------|
| 1   | MKWVTFISLL  | LLFSSAYSRG | VFRDTHKSE  | IAHRFKDLGE | EHFKGLVLIA | FSQYLQQCPF |
| 61  | DEHVKLVLNEL | TEFAKTCVAD | ESHAGCEKSL | HTLFGDELCK | VASLRETYGD | MADCCCKQEP |
| 121 | ERNECFLSHK  | DDSPDLPLK  | PDPNTLCDEF | KADEKKFWGK | YLYEIARRHP | YFYAPELLYY |
| 181 | ANKYNGVFQE  | CCQAEDKGAC | LLPKIETMRE | KVLASSARQR | LRCASIQKFG | ERALKAWSVA |
| 241 | RLSQKFPAE   | FVEVTKLVTD | LTQVHKECCH | GDLLCADDR  | ADLAKYICDN | QDTISSKLKE |
| 301 | CDKPLLEKS   | HCIAEVEKDA | IPENLPPLTA | DFAEDKDVK  | NYQEAADAF  | GSFLYEYSRR |
| 361 | HPEYAVSVLL  | RLAKEYEATL | BECCAADDPH | ACYSTVDFKL | KHLVDEPQNL | IKQNCQFEK  |
| 421 | LGEYGFQNAL  | IVRYTRKVPQ | VSTPTLVEVS | RSLGKVGTRC | CTKPESERMP | CTEDYLSLIL |
| 481 | NRLCVLHEKT  | PVSEKVTKCC | TESLVNRRPC | FSALTPEDEY | VPKAFDEKLF | TFHADICTLP |
| 541 | DTEKQIKQT   | ALVELLKHKP | KATEEQLKTV | MENFVAFVDK | CCAADDKEAC | FAVEGPKLVV |
| 601 | STQTA       |            |            |            |            |            |

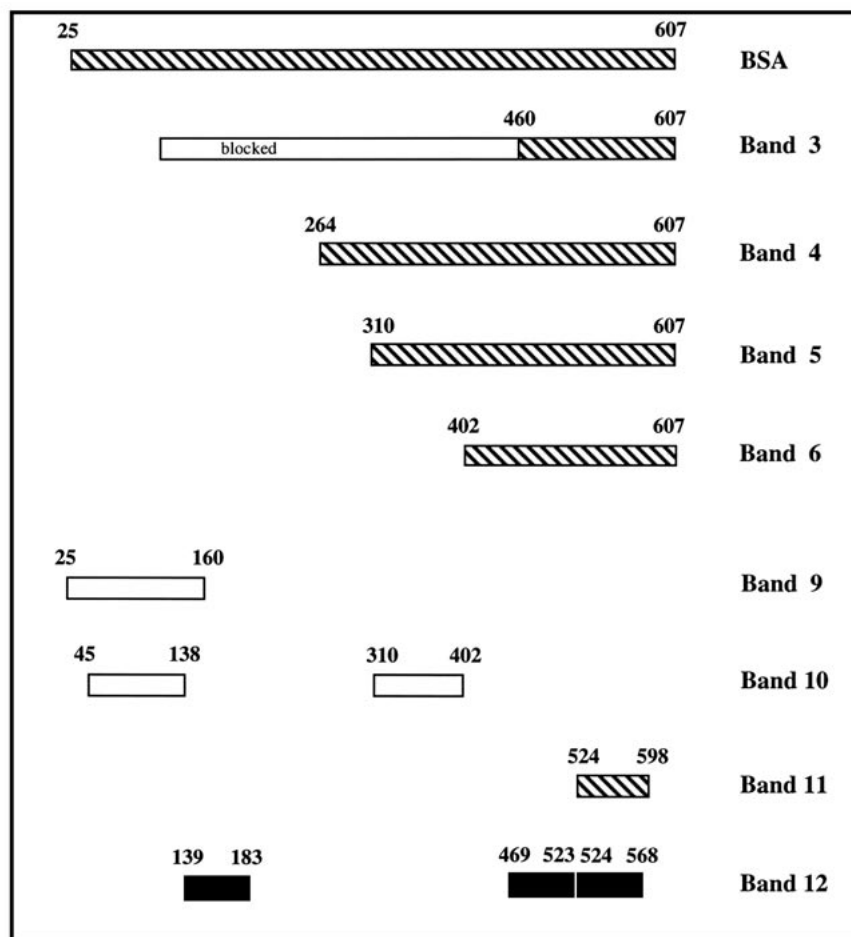


**Fig. 4.** Amino acid sequence of BSA precursor (top panel) and BSA tryptic fragments identified by automatic sequencing and positive responses obtained using human circulating IgEs (bottom panel). □ = Negative response; ▨ = positive response; ■ = peptides co-running in a positive band.

considered. The mouse IgGs were capable of binding 11 bands separated in SDS-PAGE after limited tryptic proteolysis; positive reactions were observed with polypeptides placed both at the NH<sub>2</sub>-terminal and at the HOOC-terminal portion of the BSA sequence. Human IgEs recognized only those fragments deriving from the HOOC-terminal portion of the protein. Considering the MW of the immunoreactive polypeptides, we can state that fragment 6, containing the sequence aa 402–607 and with a MW of 23.2 kD, was the shortest polypeptide recognized by IgEs

from all the subjects included in this study. On the other hand, the serum from two subjects produced immunoreactive responses also with bands 11 and 12.

Band 11 contained only one polypeptide (aa 524–598, MW 8.5 kD), while band 12 included three fragments: aa 139–183 (MW 5.4 kD), aa 469–523 (MW 6.3 kD) and aa 524–568 (MW 5.2 kD).

On the basis of the data presented above, the sequence aa 524–598 can be identified as an epitopic area for human species, even if the IgEs from some allergic sub-

**Table 2.** Sequence alignment of serum albumins from different animal species (BSA aa 520–542) and positive responses obtained with immunoblotting

| Animal species | Amino acid sequence |     |     |     |          |     |          |          |          |          |     |     |     |     |     |     |          |     |     |     |          |          |          |       | Identity with HSA % | Reacting sera <sup>1</sup> , % |
|----------------|---------------------|-----|-----|-----|----------|-----|----------|----------|----------|----------|-----|-----|-----|-----|-----|-----|----------|-----|-----|-----|----------|----------|----------|-------|---------------------|--------------------------------|
|                | 520                 | 521 | 522 | 523 | 524      | 525 | 526      | 527      | 528      | 529      | 530 | 531 | 532 | 533 | 534 | 535 | 536      | 537 | 538 | 539 | 540      | 541      | 542      |       |                     |                                |
| Cow [9]        | Y                   | V   | P   | K   | <b>A</b> | F   | <b>D</b> | <b>E</b> | <b>K</b> | <b>L</b> | F   | T   | F   | H   | A   | D   | I        | C   | T   | L   | <b>P</b> | <b>D</b> | <b>T</b> | 65.2  | 100.0               |                                |
| Sheep [21]     | Y                   | V   | P   | K   | <b>P</b> | F   | <b>D</b> | <b>E</b> | <b>K</b> | <b>F</b> | F   | T   | F   | H   | A   | D   | I        | C   | T   | L   | <b>P</b> | <b>D</b> | <b>T</b> | 65.2  | 100.0               |                                |
| Pig [22]       | Y                   | K   | P   | K   | E        | F   | <b>V</b> | <b>E</b> | <b>G</b> | T        | F   | T   | F   | H   | A   | D   | <b>L</b> | C   | T   | L   | <b>P</b> | E        | <b>D</b> | 69.6  | 83.3                |                                |
| Horse [23]     | Y                   | V   | P   | K   | E        | F   | <b>K</b> | A        | E        | T        | F   | T   | F   | H   | A   | D   | I        | C   | T   | L   | <b>P</b> | E        | <b>D</b> | 87.0  | 0.0                 |                                |
| Rabbit [24]    | Y                   | V   | P   | K   | E        | F   | N        | A        | E        | T        | F   | T   | F   | H   | A   | D   | I        | C   | T   | L   | <b>P</b> | E        | <b>T</b> | 91.3  | 0.0                 |                                |
| Human [25]     | Y                   | V   | P   | K   | E        | F   | N        | A        | E        | T        | F   | T   | F   | H   | A   | D   | I        | C   | T   | L   | S        | E        | K        | 100.0 | 0.0                 |                                |

Letters in bold indicate the points of difference between each sequence and HSA.

<sup>1</sup> Data from Restani et al. [8].

jects recognize the sequence only when it is enclosed in a longer fragment. The identified fragment confirmed the hypothesis presented in our previous paper [8], where we suggested as a possible epitopic area the sequence aa 520–542. In fact, in this amino acid sequence, important differences were observed between cross-reacting (bovine, ovine and pig) and non-cross-reacting (horse and rabbit) serum albumins. In table 2, the sequence aa 520–542 of serum albumins from different animal species are reported in parallel.

The number of sera containing IgEs capable of recognizing the serum albumin from different animal species is inversely dependent on the percentage of identity with human serum albumin (HSA): from 65.2% of BSA and ovine serum albumins (OSA) (the most reactive), to 87–91.3% of the less reactive serum albumins (horse and rabbit, respectively). In particular, BSA and OSA show two main areas of dishomogeneity when compared to HSA: the sequences aa 526–529 and aa 540–542. When these short sequences become more similar to that of HSA, the number of reactive sera falls rapidly. In pig serum albumin, amino acids in positions 529 and 541 change to threonine and glutamic acid, respectively, which are the amino acids found in HSA. As a consequence, the number of positive sera decreases to 83.3%. With further amino acid substitution in the cited sequences (see horse and rabbit sequences), the cross-reactivity with BSA is lost, as shown by the negative responses obtained with the sera of all allergic children included in our study.

Our results are partially in agreement with data reported by other authors. Most papers that describe the antigenic activity of fragments obtained after limited hydrolysis of BSA include at least a sequence containing aa 520–542,

more precisely: (1) the tryptic fragment aa 377–581, with at least an epitopic site in the sequence aa 504–581 [13]; (2) the tryptic fragment aa 377–571 [15], and (3) the fragments aa 307–582 and aa 377–582, produced by peptic and tryptic proteolysis, respectively [17, 18].

The epitopic sites indicated above support our findings suggesting aa 402–607 as the shortest sequence capable of binding human IgEs of all allergic subjects included in this study. The most critical sequence in this fragment seems to be aa 524–542, even if this peptide must be included in a longer sequence to be recognized by antibodies.

The same phenomenon has been described by Ferguson et al. [17], whose studies demonstrated that the fragment aa 505–546, obtained with BrCN, was a low immunogen compared to the tryptic fragment aa 377–582.

For murine antibodies, the epitopic area includes other sequences of BSA (bands 9 and 10) confirming previous data obtained using animal antibodies.

In conclusion, the results presented in this study indicate that the epitopic sites of an antigenic protein can change when different species are studied. This consideration explains why data about the antigenic characteristics of BSA were not always in agreement, confirming the importance of the different experimental conditions. Finally, researchers must be aware that data obtained with antibodies from animal species cannot be directly extrapolated to the behavior of human IgEs.

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