

Comparison of Human IgE-binding Soya Bean Allergenic Protein Gly m I with the Antigenicity Profiles of Calf Anti-soya Protein Sera

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In the food, particularly the feed, industry, large quantities of soya bean protein products are used for the formulation of end-products destined for human or animal consumption. These basic ingredients and the final end products often have to fulfill specific requirements regarding the presence of antinutritional compounds (ANCs) and their allergenic potential. Therefore, a special need exists for the identification and characterization of ANCs and the causative factors behind allergy and hypersensitivity. This paper describes the results of a comparative study of a previously identified unknown antigenic soya bean P22-25 protein with the first, recently described Gly m I (or P34) soya bean allergen. Sodium dodecyl sulphate polyacrylamide gel electrophoresis and Western blotting analysis were carried out using calf anti-soya protein sera and a murine anti-Gly m I (or P34) monoclonal antibody in order to detect the antigenic soya bean P22-25 protein and the allergen Gly m (or P34) respectively. The results showed that these proteins were different. In addition, a number of industrial soya bean products were analyzed for the presence of both apparent antigenicity (essentially β -conglycinin and P22-25) using calf anti-soya protein sera and the allergen Gly m I (or P34), and both β -conglycinin, P22-25 and P34 could be detected to a varying extent in the products analyzed.

Keywords: Allergenicity, antigenicity, soya beans, Gly m I (or P34), Gly m Bd 30K

INTRODUCTION

Soya beans contain a number of antinutritional compounds (ANCs), such as protease inhibitors, lectins, and non-starch polysaccharides. These appear to be responsible for a reduced efficiency of utilization of plant proteins compared with milk proteins if they are included in the animal or human diet (Sissons & Tolman, 1991; Gueguen *et al.*, 1993). In

the feed industry, it is generally believed that the antigenicity of (particular) soya proteins contributes to the overall problem of maldigestion and gastrointestinal disturbances. Antigenic proteins have the ability to activate the immune system, which directly relates to suboptimal digestion of these proteins, enhanced maintenance requirements and loss of endogenous protein (Sissons & Tolman, 1991). The intestinal tract usually provides an effective barrier against excessive absorption of microorganisms and feed antigens normally existing in the intestinal lumen. However, during gastrointestinal infection, and also in newborn infants and animals, the mucosal barrier is less effective than in adults, and the uptake of feed or food antigens may be increased. This leads to the generation of antibodies against the absorbed antigens (Kilshaw, 1981; Taylor *et al.*, 1987) and may under particular circumstances, favour the development of an allergy through failure to develop oral tolerance. Gastrointestinal adverse responses in pre-ruminant calves fed with soya flour have been reported by Kilshaw and Sissons (1979 a,b) and Barratt *et al.* (1978, 1979), and it was assumed that the soya proteins, especially glycinin and β -conglycinin (Fukushima, 1991 a,b), were responsible for the negative effects (Sissons *et al.*, 1982; Sissons & Thurston, 1984). Using calf anti-soya protein sera obtained from orally sensitized animals, Hessing *et al.* (1993, 1994) observed that the calf antibody responses were mainly directed against β -conglycinin and an unknown low molecular weight antigenic P22-25 soya protein.

Various IgE-binding soya proteins have been reported in a number of clinical studies using sera from soya-allergic humans (Moroz & Yang, 1980; Shibasaki *et al.*, 1980; Bush *et al.*, 1988; Burks *et al.*, 1988; Ventling & Hurley, 1988; Eastman, 1989; Herian *et al.*, 1990). More recently, Ogawa *et al.* (1991) demonstrated the occurrence of the major allergen in soya beans, *Gly m Bd 30 K*, that was most strongly and frequently bound by the IgE antibodies of the sera of soya-allergic Japanese citizens. In 1993, this soya bean allergenic protein *Gly m Bd 30 K* was further characterized (Ogawa *et al.*, 1993), and was identified as the soya bean seed 34-kDa oil body associated protein or P34 (Herman *et al.*, 1990; Kalinski *et al.*, 1990). This protein showed considerable sequence similarity to the thiol proteinases of the papain superfamily, and demonstrated a remarkable homology of about 30% with the house dust mite allergenic thiol proteinase *Der p I*, from *Dermatophagoides pteronyssinus*. It was recommended that *Gly m Bd 30 K* (or P34) should be named *Gly m I*, the first characterized allergenic protein in soya beans (Ogawa *et al.*, 1993).

A direct relationship between antigenicity measurements *in vitro* and allergic responses *in vivo* is difficult to demonstrate. The present study was performed to examine the possible identification of the previously recognized unknown low molecular weight antigenic P22-25 soya protein (Hessing *et al.*, 1993, 1994) with the *Gly m I* (or P34) soya bean allergen.

MATERIALS AND METHODS

Materials

Protein A-Sepharose, DEAE-Sepharose CL-6B and Sephacryl S-200 media were obtained from Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden). Pre-stained molecular weight markers were from BioRad Laboratories (Richmond, CA, USA). Bovine serum albumin (BSA) and dithiothreitol (DTT) were obtained from Sigma Chemical Co. (St Louis, MO, USA). The soya bean products used in this study (designated 1-9) were the kind gifts of several industrial companies, and included soya bean protein products with an expected high antigenicity (4), a heat-treated soya meal with an expected intermediate antigenicity (7), soya concentrates with an expected low (6) or high (1) antigenicity, an ethanol-extracted soya protein concentrate with mild heat treatment with an expected intermediate antigenicity (8), soya isolates with an expected low (3) or high (5) antigenicity, an enzyme-modified soya flour with an expected low antigenicity (2) and an enzyme-treated soya protein isolate with an expected low antigenicity (9) (Hessing *et al.*, 1996). The samples had undergone different forms of processing, although the precise nature of the processing was not disclosed. Each soya sample was either supplied as flour or milled prior

to analysis to a particle size of ≤ 0.5 mm using a laboratory mill (Retsch, Haan, Germany, 0.5 mm). All other chemicals were of the best grade available.

Soya Bean Protein Fractions

A crude P22-25 antigenic soya protein fraction was prepared from defatted soya bean meal (Cargill 200/90). Soya bean meal (50 g) was homogenized with 1 l of 30 mM-Tris-HCl, 10 mM-DTT, pH 8.0 at room temperature for 1 h and the homogenate was centrifuged at $10\,000 \times g$ for 45 min. The supernatant was incubated with 40% ammonium sulphate at room temperature and the precipitate was collected by centrifugation at $30\,000 \times g$ for 30 min. The precipitate was dissolved in a minimum volume of 30 mM-Tris-HCl, 10 mM-DTT, pH 8.0, dialyzed against the same buffer for 20 h at 4°C and frozen at -20°C. *Gly m I* (or P34) was purified essentially as described by Kalinski *et al.* (1992) with two additional steps using DEAE-Sepharose CL-6B anion exchange chromatography and Sephacryl 200 gel filtration (Ogawa *et al.*, 1993). The purified P34 preparations were lyophilized and frozen at -20°C.

Antibodies

A mouse anti-P34 monoclonal antibody (F5; Ogawa *et al.*, 1993) producing hybridoma was prepared by fusion of Balb/c mouse spleen cells with P3U1 myeloma cells, and was injected intraperitoneally into pristane-primed mice for the production of antibody-rich ascites fluids. The murine IgGs were isolated by ammonium sulphate precipitation and protein A chromatography. The anti-P34 monoclonal antibody F5 was of the IgG_{2a} subclass and recognized a sequential epitope on P34 (Tsuji *et al.*, 1993). The concentration of the purified monoclonal antibodies was determined by the method of Lowry *et al.* (1951) using BSA as the standard. Calf anti-soya protein antibodies were obtained from young calves, which had been orally sensitized by feeding them with untreated soya beans as described previously (Hessing *et al.*, 1993, 1994). Polyclonal rabbit anti-calf and goat anti-mouse peroxidase-conjugated antibodies were obtained from Nordic (Tilburg, The Netherlands).

Electrophoretic and Immunochemical Techniques

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially according to Laemmli (1970) using Mini-Protean II Ready Gels (BioRad Laboratories, Richmond, CA, USA). The gels were either stained with Coomassie brilliant blue R-250 or subjected to Western blotting (Towbin *et al.*, 1979). The Immobilon-P membranes (Millipore, Molsheim, France) were blocked with 3% (w/v) non-fat dry milk (Protifar, Nutricia, The Netherlands) in 50 mM-Tris-HCl, pH 7.4, 150 mM-NaCl, incubated with either mouse anti-P34 monoclonal antibody F5 or with calf anti-soya protein antibodies and visualized with peroxidase-conjugated antibodies. Purified allergen P34, crude P22-25 antigenic soya protein fraction and the soya bean products (5 mg ml⁻¹) were boiled for 20 min in 1% (w/v) dithiothreitol, 63 mM-Tris-HCl, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue, 20% (v/v) glycerol, pH 6.8. The mixture was then placed for 30 min in an ultrasonic bath and centrifuged for 15 min at 10 000 rpm (Sigma 202 MC).

RESULTS AND DISCUSSION

The electrophoretic pattern of the purified allergen *Gly m I* (or P34) and a crude P22-25 antigenic soya protein fraction is shown in Figure 1. The identification of the unknown P22-25 antigenic soya protein with P34 could not be made, as the Western blotting analysis using mouse anti-P34 monoclonal antibody F5 (Figure 1(b)) and calf anti-soya protein antibodies (Figure 1(c)) clearly revealed that these soya proteins were definitively different. Figure 1(b) showed that the allergen *Gly m I* (P34) was present in the extracted soya

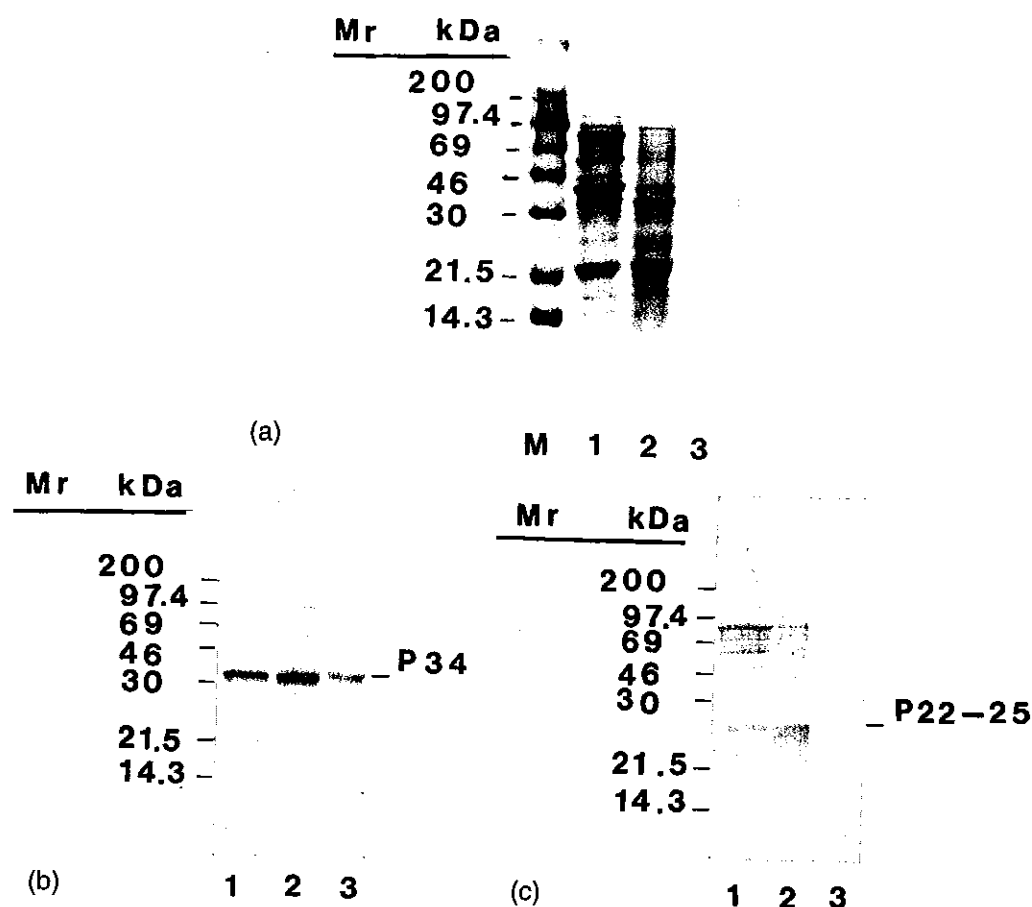


FIG. 1. SDS-PAGE (15%) (a) of soya bean meal (lane 1), a crude P22-25 antigenic soya protein fraction (lane 2) and purified allergen *Gly m I* (P34; lane 3). Western blotting analysis using (b) mouse anti-P34 monoclonal antibody F5 (2 µg ml⁻¹) and (c) calf anti-soya protein antiserum (1/50 diluted). M, pre-stained molecular mass marker proteins.

proteins of the analyzed soya bean meal (lane 1). In addition, P34 was found in the crude P22-25 antigenic soya protein fraction (lane 2) obtained after 40% ammonium sulphate precipitation (see Materials and Methods). This is not surprising as 40% ammonium sulphate precipitation is also the first step for P34 purification (Ogawa *et al.*, 1993). Figure 1(c) demonstrates that P22-25 antigenic soya protein is present in both the extract of the analyzed soya bean meal (lane 1) and the 40% ammonium sulphate precipitate (lane 2), but that the calf anti-soya protein antibodies exhibited no significant immunoreactivity with the isolated allergen P34. This latter observation indicates that the isolated allergen P34 is not recognized by calf anti-soya protein antibodies, which may suggest that P34 is not an allergenic protein for calves. However, it should be noted that the predominantly IgG subclass responses in the calf sera are not comparable with those of previous studies, in which soya bean allergenic P34 was most strongly and frequently recognized by IgE antibodies in the sera of soya bean-sensitive patients with atopic dermatitis (Ogawa *et al.*, 1991). The binding characteristics of these previously reported human IgE and calf Ig antibodies to the various soya protein fractions are summarized in Table 1.

In addition, a total of nine soya samples obtained from various industrial sources, including samples with expected low antigenic levels (based on the processing method

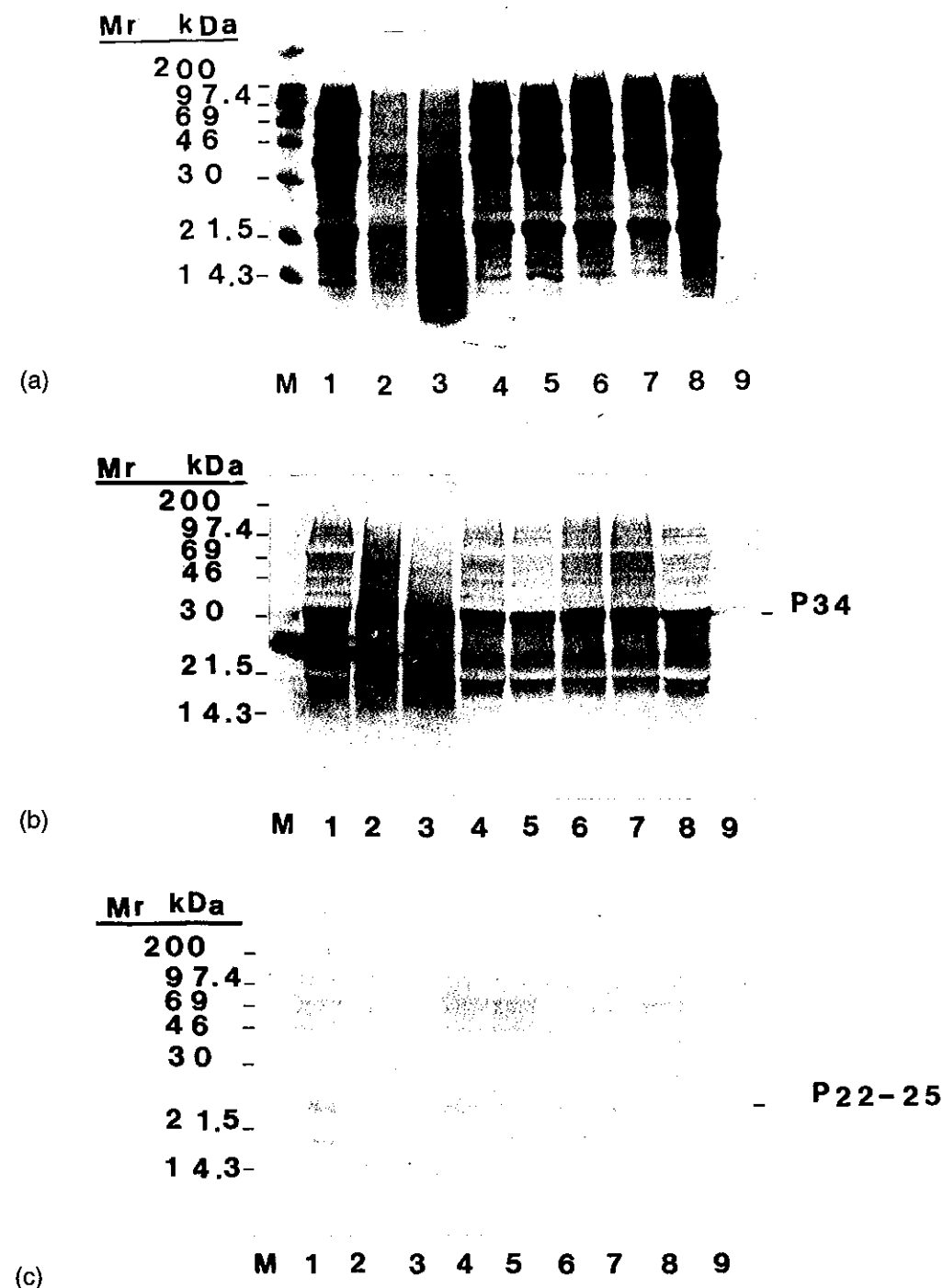


FIG. 2. SDS-PAGE (15%) of (a) soya bean products 1-9 (lanes 1-9) and Western blotting analysis using (b) mouse anti-P34 monoclonal antibody F5 (2 µg ml⁻¹) and (c) calf anti-soya protein antiserum (1/50 diluted). M, pre-stained molecular mass marker proteins.

TABLE 1. Binding characteristics of human IgE and calf Ig antibodies to soya protein fractions

Molecular weight (kDa)	Assignment of soya protein fraction	Binding of human IgE ^a	Binding of calf Ig ^b
80-68	β -Conglycinin (α/α' -subunit)	++	+++
67-60	β -Conglycinin (β -subunit)	++	+++
50-35	7S-Globulins	+	+
38-35	Glycinin (acidic subunit)	+	+
34	Gly m I (p34)	+++	-
25-22	P22-25	+	+++
21	Kunitz trypsin inhibitor	+	+

^aModified from Ogawa *et al.* (1991).^b+++ , strong; ++ , moderate; + , weak and - , no reactivity of the antibodies with the soya protein fraction.

used), were analyzed for the presence of the allergen P34 and the low molecular weight antigenic soya bean P22-25 protein using SDS-PAGE and Western blotting analysis (Figure 2). Figure 2(a) shows the electrophoretic profiles of the proteins extracted from the various soya bean products after Coomassie blue staining. It can be seen that in soya product 3 (soya isolate) and particularly products 2 and 9 (an enzyme-modified flour and an enzyme-treated soya protein isolate), the soya proteins are degraded to smaller peptides, whereas the other products contain rather intact protein bands. Western blotting analysis using a mouse anti-P34 monoclonal antibody F5 (Figure 2(b)) demonstrated that apart from soya product 9, all soya products contained the P34 allergen, although soya samples 2 and 3 showed a slightly decreased immunoreactivity. Figure 2(c) shows the results of Western blotting analysis using calf anti-soya protein antibodies. For soya samples 2, 3 and 9, no significant immunoreactivity with calf anti-soya protein antibodies was observed, which is in agreement with their suspected low antigenic character. For the other soya samples, a typical antigenicity profile with immunoreactive β -conglycinin subunits (Fukushima, 1991 a,b) and the unknown low molecular weight P22-25 soya protein (Hessing *et al.*, 1994) commonly seen using the calf anti-soya sera was obtained. For the suspected low antigenic soya sample 6 (soya concentrate) and soya sample 7 (a heat-treated soya meal), a slight decrease in the immunoreactivity of the β -conglycinin subunits was observed, whereas the immunoreactivity of the antigenic P22-25 protein remained intact.

In conclusion, this study demonstrates that a previously identified unknown antigenic soya bean P22-25 protein (Hessing *et al.*, 1993, 1994) is different from the first recently described Gly m I (or P34) soya bean allergen (Ogawa *et al.*, 1991, 1993). Both the antigenic P22-25 and allergenic P34 soya proteins could be detected in a number of industrial soya bean products. However, although the effect of the presence of these proteins in animal nutrition products are difficult to predict, it is justifiable to conclude that attention may be paid to allergen P34-containing soya products if they are to be used for human consumption.

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Modification of Nitrite Production and Phagocytosis of Thioglycollate-elicited Mouse Peritoneal Macrophages by Bovine Casein Digests

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The effect of bovine milk casein digests on the ability of thioglycollate-elicited mouse peritoneal macrophages to produce nitrite and to ingest latex beads was investigated. Intact α s₁-casein and κ -casein significantly inhibited nitrite production by the macrophages. The inhibitory activity of the latter was little influenced by trypsin or chymotrypsin digestion, while that of the former decreased with increasing proteinase digestion time. In contrast, pepsin digestion changed the inhibitory activity of α s₁-casein and κ -casein into an enhancing activity. Intact β -casein, however, had an enhancing effect on nitrite production, and its activity increased noticeably when the protein was digested with pepsin. The enhancing activity of pepsin digests of each casein component reduced when the digest was treated with carboxypeptidase A. Moreover, a trypsin or chymotrypsin digest of α s₁-casein and κ -casein inhibited ingestion of latex beads by the macrophages, while a pepsin digest of each casein component enhanced it. These results suggest that the neonate ingestion of infant formula made with bovine milk might reduce resistance to infection via suppression of macrophage functions, such as antimicrobial nitrogen intermediate production and phagocytosis in their gut mucosa, since it is known that gastric secretion and pepsin activity are weak in neonates and the major portion of the caseins ingested by neonates leaves the stomach after minimal digestion.

Keywords: Casein digest, peritoneal macrophages, nitrite production, phagocytosis, regulation of macrophage function

INTRODUCTION

Milk is a complex mixture of proteins, carbohydrates, lipids, minerals, vitamins and trace elements secreted by the mammary gland for the purpose of neonatal nutrition. Milk is also considered to be a source of biologically active components with important physiological roles, as some milk proteins and their digests have been demonstrated to possess biological activities (Fiat & Jollès, 1989). The neonate is immature in certain immunological functions