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Enzyme-linked Immunosorbent Assay and Immunoblotting Using IgY Antibodies Against Soybean Glycinin A

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ABSTRACT

Using IgY antibodies obtained from egg yolk of immunized chickens, an enzyme-linked immunoassay (ELISA) and an immunoblotting procedure has been developed for specific determination of soy protein. The procedure for antigen preparation provides a rapid method for the isolation of SDS-denatured glycinin A from polyacrylamide gels that could be used directly for immunization.

ELISA with SDS-denatured soy protein isolate was linear in the range 0·5–256 μg/ml and covers the expected levels of soy protein in simulated milk and milk products. Immunostaining of SDS–PAGE blots allowed the detection of glycinin A at nanogram levels.

It is claimed that the antigenic status of SDS-denatured glycinin A is independent of food processing conditions and that the specific immunoreaction is controlled by high affinity IgY antibodies against the SDS-denatured protein conformation.

INTRODUCTION

Soy protein is one of the most important and frequently used vegetable protein additives in food products. Soy protein preparations have been used as protein substitutes or extenders in meat products, soups, infant formulae and dairy imitation products (e.g. simulated milk, cheese,

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yoghurt). The legal regulations in many countries demand analytical methods to establish the protein composition of the final product and to reduce the chance of adulteration. The increasing market of new dairy-like products must be accompanied especially by the development of effective methods both for detection and quantitation of soy protein additives.

Several analytical approaches have been employed for soy protein analysis, including high-performance liquid chromatography (Ashoor & Stiles, 1987), peptide fragment analysis (Medina & Phillips, 1982), amino acid pattern (Elenbaas *et al.*, 1985), electrophoresis (Armstrong *et al.*, 1982; Olsman *et al.*, 1985; Hewedy & Smith, 1989) and immunoassays (Hitchcock *et al.*, 1981; Ravestein & Driedonks, 1986; Rittenburg *et al.*, 1987; Medina, 1988; Ventling & Hurley, 1988; Hewedy & Smith, 1990). The main advantage of immunochemical methods is their high specificity, so that the analyte may be determined without isolation. Furthermore, immuno-assays allow a large sample throughput. A major problem for the development of practicable food immunoassays is the selection of antigen which retains its specificity and immunochemical reactivity after the technological treatments.

Immunoreagents containing IgY antibodies can be easily prepared from the eggs of immunized laying hens by a non-invasive technique. It has been shown recently that IgY antibodies are suitable for the immunochemical determination of soy and milk protein (Eichler & Rubach, 1986; Meisel, 1990) in food.

This paper describes an ELISA and an immunoblotting procedure for the detection and determination of soy protein using IgY antibodies that are specific for sodium dodecyl sulphate (SDS)-denatured acidic polypeptides of soy glycinin.

MATERIALS AND METHODS

Antigen preparation

Soy protein isolate (Purina 610; Ralston Purina), containing 82·2% protein (based on amino acid analysis according to Meisel & Frister, 1988), was dispersed at a concentration of 20 mg ml^{-1} in sample buffer (37·5 mmol l^{-1} Tris-HCl, pH 8·8) containing 4% sodium dodecyl sulphate (SDS) and 0·15% dithiothreitol (DTT; Sigma) and the soy protein was dissolved during heating at 100°C for 5 min.

For preparative SDS electrophoresis (SDS-PAGE), $20 \times 5\,\mu l$ of

solutions of the SDS-denatured soy protein isolate were applied to the sample slots (7 × 2 mm) of a thin layer (0·5 mm) polyacrylamide gel with a 10 mm stacking gel (4% T, 3% C) and a 90 mm separating gel (12% T, 3% C) containing 0·1% SDS. The gel preparation and composition of buffers were described by Meisel & Carstens (1989). After a pre-run at 15 mA to concentrate the samples in the stacking gel, electrophoresis was performed at 30 mA constant current until the marker dye (bromphenol blue) reached the anode side.

The protein bands were visualized in the gel by potassium dodecyl sulphate (KDS)-staining (Prussak *et al.*, 1989): following electrophoresis, the gel was first rinsed in cold water and then soaked for 10 min in 0·25 mol l^{-1} ice-cold KCl. This procedure results in potassium precipitation of dodecyl sulphate bound to protein and thus the formation of white protein bands. The main band of glycinin A (Fig. 1) was excised as a continuous gel slice and homogenized in 5 volumes of PBS, pH 7·2, by passing it vigorously four times through a 1·2 mm-bore needle fitted to a 5-ml syringe.

Three preparative runs with KDS-staining were performed to obtain the final antigen preparation of glycinin fraction A.

Preparation of IgY immunoreagent

Hens (Rote Rhodeländer hybrids) were housed in individual cages for immunization and egg production. For primary immunization, the antigen preparation containing the glycinin fraction A was diluted 1 + 1 (v/v) in a solution of 0·2% SDS and 0·15% DTT (w/v), heated at 100°C for 5 min and then emulsified with an equal volume of Freund's complete adjuvant (Sigma). One millilitre of this emulsion containing about 65 μg protein, was injected into the breast muscle (4 sites). Five booster injections with 1–2 ml of the same antigen preparation, but with Freund's incomplete adjuvant instead, were given at 1, 3, 5, 30 and 31 weeks after the first injection.

IgY antibodies were isolated from egg yolk by the precipitation technique of Polson *et al.* (1980) using polyethylene glycol (PEG 6000). A concentration of 3·5% PEG caused the lipids and vitellin to separate; the IgY-pellet obtained after precipitation in a final concentration of 12% PEG was then redissolved in PBS in a volume equal to 1/10 of the original yolk volume yielding a protein concentration of 100–150 mg ml^{-1} (Coomassie G-250 protein reagent according to Bradford, 1976). The resultant immunoreagent was precipitated with ammonium sulphate at 50% saturation and stored at −20°C (Montoya & Castell, 1987).

ELISA procedure

Standards were prepared as follows: 12 mg of the soy protein isolate was dispensed in 5 ml PBS, pH 7·2, containing 1% SDS and 0·15% DTT and then heated at 100°C for 5 min. A portion (3·2 ml) of this solution was diluted to a final volume of 25 ml with PBS. This solution was serially 2-fold diluted with PBS/0·1% SDS, giving concentrations of soy protein from 256 $\mu g/ml^{-1}$ to 0·5 $\mu g\ ml^{-1}$.

The specificity of the ELISA was tested using skim milk powder, casein (Biogen, BMI), α-lactalbumin (Sigma), β-lactoglobulin (Sigma) and protein isolate of faba bean (kindly provided by E. Schmandke, Central Institute of Nutrition, Potsdam-Rehbrücke), pea (Grindsted), wheat (Amylum NV), potato (Emsland-Stärke), rape and alfalfa (Rhône-Poulenc). These samples were prepared in the same way as the soy protein standard, based on their protein content as determined by the Kjeldahl method (N-to-protein conversion factor 5·7).

Samples of pasteurized cows' milk, soy drink (Nuxo) and mixtures of bovine milk and soy drink were diluted 1:10 to 1:1000 with PBS/0·1% SDS and then heated at 100°C for 5 min. Samples and standards were analysed directly by ELISA without removal of SDS.

The indirect ELISA was developed using a modification of the assay system described in a previous paper (Meisel, 1990). Optimal assay conditions were found in preliminary tests using several combinations of antigen concentrations coated on the wells, IgY-immunoreagent and enzyme conjugate, respectively. The procedure is outlined in seven steps:

(1) coating 96 well plate (Immulon, Dynatech) overnight at 37°C with 100 ng/well soy protein isolate in 100 μl carbonate coating buffer, pH 9·8;
(2) concurrent but separate incubation of 200 μl sample with 200 μl IgY-immunoreagent (diluted 1:50 in PBST) in polypropylene tubes (System Micronic; Flow Lab.) overnight at 4°C;
(3) plate wash (twice) with 200 μl/well PBST followed by blocking well with 200 μl/well 1% gelatine in PBS for 60 min at 37°C;
(4) plate wash (twice) as before followed by addition of 100 μl/well of sample-immunoreagent mixture (step 2) for 90 min at 37°C;
(5) plate wash (four times) as before followed by addition of 100 μl/well rabbit anti-chicken-IgG peroxidase conjugate (Nordic Immunological Lab.) diluted 1:5000 in PBST containing 3% BSA, and then incubation for 90 min at 37°C;
(6) plate wash as before, followed by incubation with 100 μl/well

ABTS-H_2O_2 substrate solution (ABTS:2,2′-azino-di(3-ethylbenzothiazoline-6-sulfonic acid); Sigma), pH 4·0, for 15 min at room temperature; the enzyme-substrate reaction was terminated with 50 μl/well NaF stop solution;

(7) absorbance measurement at 410 nm against the substrate blank well.

Samples and standards were run in duplicate and were arranged in rows. In addition, each ELISA plate had at least two maximum binding values (no competing antigen in step 2) and two zero binding values (blanks, no antigen bound to the wells in step 1). The unspecific absorbances of the blanks were below 0·01 and the mean was subtracted from the samples and standard values.

Immunoblotting

After SDS-PAGE of SDS-denatured samples as described above, proteins were transferred to nitrocellulose (BA 83 or BA-S 83, 0·2 μm; Schleicher & Schüll) by electroblotting (Multiphor II Nova Blot; LKB) at 0·8 mA cm^{-2} for 2 h in a continuous buffer system (24 mmol l^{-1} Tris-HCl, pH 8·3, 192 mmol l^{-1} glycine) or a discontinuous buffer system (anode solution 1 and 2, pH 10·4, 300 and 25 mmol l^{-1} Tris, respectively; cathode solution, pH 7·6, 40 mmol l^{-1} 6-amino-*n*-hexanoic acid) containing 20% (v/v) methanol.

Protein blots were sequentially subjected to:

(1) incubation in 0·2 mol l^{-1} NaOH for 5 min and then rinsed briefly in water;
(2) blocking with 5% BSA in TBST (20 mmol l^{-1} Tris-HCl, pH 7·5, 100 mmol l^{-1} NaCl, 0·05% Tween 20) for 30 min;
(3) a wash in TBST for 1 min;
(4) incubation with diluted (1:100) IgY antiglycinin immunoreagent in TBST for 2 h;
(5) three washes in TBST for 5 min each;
(6) incubation with diluted (1:10 000) peroxidase conjugate (see ELISA) in TBST/1% BSA for 2 h;
(7) three final washes in TBST for 5 min each (steps (2) to (7) were performed with constant agitation in 25 ml solution);
(8) tetrazolium method for peroxidase staining was applied using phenol as an enzyme substrate and NADH as a reducing agent for Nitro Blue tetrazolium (NBT; Sigma) to form formazan (Taketa, 1987). A 20 ml staining solution consisting of 40 mg NADH, 8 mg

phenol, 6 mg NBT and 0·02% (w/v) H_2O_2 in 50 mmol l^{-1} phosphate buffer, pH 7·0, was prepared as described by Taketa (1987). Color development was carried out at 20–25°C protected from light for 30 min. The stained blots were rinsed in water and air dried.

The effect of the anionic detergent, SDS, on antigen-antibody interaction and cross-reaction was examined using different concentrations of SDS from 0·01 to 0·2% in step (4).

RESULTS

The protein fractions of soy protein isolate were separated by preparative SDS-PAGE (Fig. 1) and the gel band containing the acidic polypeptides A_1, A_2, and A_4 of soy glycinin could be used directly to raise antibodies against this protein fraction. Chickens were immunized with SDS-denatured glycinin $A_{1,2,4}$ and the IgY antibody activity in the egg yolk was monitored by ELISA between 3 weeks and 60 weeks following the first antigen injection (Fig. 2). The IgY activity started to increase significantly after primary immunization and gradually declined if no further booster injection was given.

Standard curves obtained by ELISA with SDS-denatured soy protein

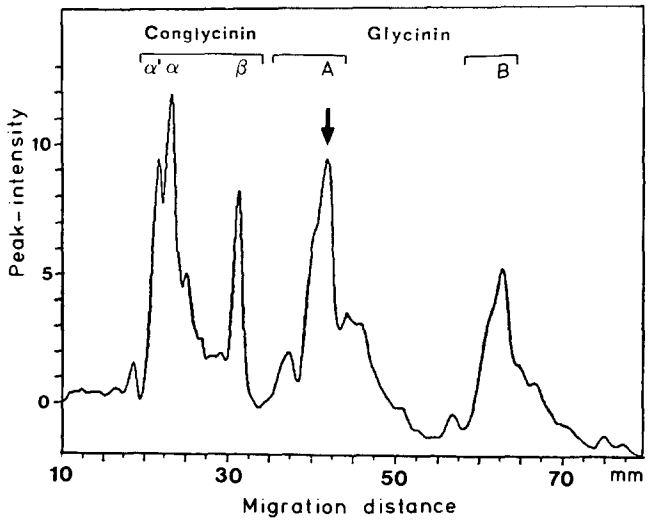

Fig. 1. Densitometric scan of SDS-PAGE patterns of soy protein isolate stained with Coomassie Blue. The different polypeptides were identified using the nomenclature of Iyengar & Ravestein (1981). The fraction of soy glycinin A (indicated by arrow) containing the acidic polypeptides A_1, A_2, and A_4 was isolated after KDS-staining.

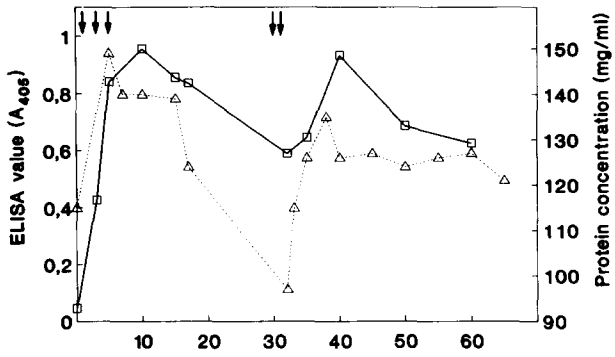

Fig. 2. Typical changes of IgY antibody activity during immunization period (weeks after first injection). The arrows indicate when the chicken was injected with SDS-denatured glycinin A fraction isolated by SDS-PAGE. IgY activity is expressed as ELISA absorbance (□) obtained with 1 μg/ml soy protein standard and as protein concentration (△) in the IgY immunoreagent.

isolate were linear in the range 0·5–256 μg ml^{-1} (Fig. 3). The range of intra-assay CV of the standard concentrations was 2·1–5·6%; inter-assay CV ranged from 6·7 to 15%. The assay was designed to cover the expected levels of soy protein in foods, especially in milk and milk products. Experiments to determine soy protein in spiked milk samples demonstrated the absence of matrix effects, since identical standard curves were obtained when soy protein isolate was diluted in assay diluent (PBS) as well as in pasteurized milk (Fig. 3). Furthermore, dose response curves of soy drink added to pasteurized milk at spiking levels of 1 to 32% were parallel to soy protein standard curves (Fig. 3), indicating that the same epitopes are being recognized in the proteins from soy drink samples as in the soy isolate standard. Table 1 shows that the ELISA was highly specific for soy protein; low ELISA values corresponding to more than 90% bound are attributed to an unspecific response of the non-soy proteins.

In order to obtain information about possible cross-reactions with non-soy proteins, the IgY immunoreagent was examined by an immunoblotting technique. As revealed by the immunoperoxidase staining of the SDS-PAGE blots, antiglycinin A-IgY can bind to epitopes on glycinin A from different soy products, mixtures of soy drink with cows' milk and to epitopes on protein fractions of faba bean and pea (Fig. 4). The SDS-electrophoretic mobility of the cross-reacting proteins did not correspond to the glycinin A band. No cross-reaction was detected by immunostaining of proteins from cows' milk and from isolates of wheat, potato, rape and alfalfa (plant proteins not shown in

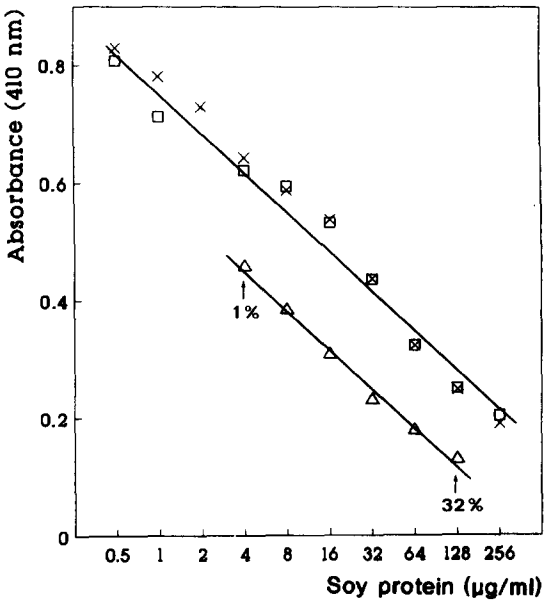

Fig. 3. Response curves of ELISA for SDS-denatured soy glycinin fraction A using IgY immunoreagent: standard (soy protein isolate) in assay diluent (PBS), (□); standard in diluted (1:100) bovine milk, (×); mixtures of 1%, 2%, 4%, 8%, 16% and 32% soy drink in bovine milk, (△); the spiked samples were diluted 1:100 for ELISA. The maximum binding (no competing antigen) gave an average absorbance value of 0·927 (n = 6).

Fig. 4). After immunoblotting of skim milk powder, weakly stained diffuse bands were occasionally observed in the region of bovine α- and β-caseins, but only with IgY preparations obtained more than 10 weeks after primary immunization.

The detergent SDS, at concentrations from 0·01 to 0·05% (immunoblotting, step 4), had no detectable effect on the specific interaction of IgY with glycinin A. However, SDS at a concentration of 0·05% markedly reduced the non-specific binding to faba bean and completely inhibited the cross-reaction with pea proteins. At concentrations of 0·2% SDS no protein bands at all could be detected on immunoblots. When the original polyacrylamide gel was Coomassie-stained after blotting, no distinct protein band could be detected. Thus, the blotting procedure, using a continuous or discontinuous buffer system, removed all protein from the gel.

DISCUSSION

Hen's egg yolk is a convenient source of large quantities of specific and high affinity IgY antibodies. Moreover, IgY antibodies can be purified to

TABLE 1
Response of Indirect ELISA to Non-Soy Proteins Compared to Soy Protein (Standard)

Sample	*% Bound (B/B_0)*[a]
Soy standard	56·0[b]
Faba bean	100·9
Pea	100·2
Wheat	100·8
Potato	99·9
Rape	95·3
Alfalfa	95·8
Skim milk powder	92·9
Casein	91·2
β-Lactoglobulin	97·0
α-Lactalbumin	101·5

[a]B = absorbance at a protein concentration of 10 μg/ml; B_0 = absorbance at maximum binding (100% bound), i.e. no competing protein present in the incubation mixture.
[b]Measurements were run in duplicate and the results are the mean from two independent assays.

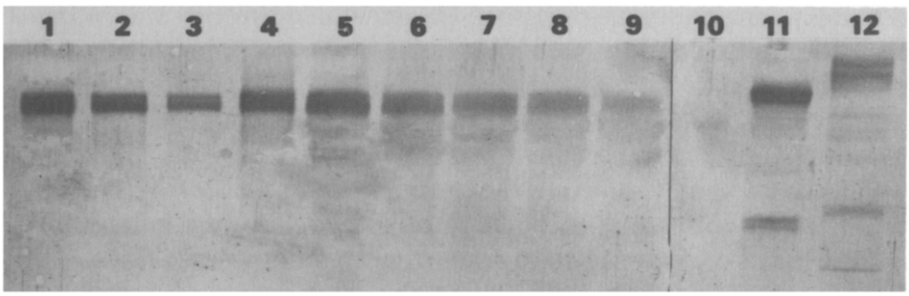

Fig. 4. Immunoblot of commercial soy products and mixtures of soy drink in bovine milk. The samples were separated by SDS-PAGE and transferred to nitrocellulose membranes for immunolocalization of protein bands using IgY immunoreagent: (1) soy protein isolate, 1·5 μg; (2) 0·75 μg; (3) 0·375 μg; (4) soy flour, 3 μg; (5) mixture of 10% soy drink in pasteurized bovine milk; (6) 5% soy drink; (7) 3% soy drink; (8) 2% soy drink; (9) 1% soy drink; (10) skim milk powder, 3 μg; (11) faba bean protein isolate, 3 μg; (12) pea protein isolate, 3 μg.

near homogeneity by simple techniques and used for different kinds of immunoassays. Regarding the application of IgY antibodies in food analysis, ELISA using IgY against bovine lactoferrin (Meisel, 1990), casein (Otani *et al.*, 1989, 1991), α-lactalbumin and β-lactoglobulin (Stapf

et al., 1991) as well as soy conglycinin (Eichler & Rubach, 1986) have been described recently.

The immunochemical detection of soy protein in dairy-like products demands specific immunoreagents against defined epitopes on soy proteins. In this study, particular attention has been given to a major problem associated with (1) differences in immunogenicity of individual soy protein fractions, and (2) altered immunoreactivity of the proteins due to denaturating conditions in manufacturing processes.

Glycinin A was considered as the best soy protein marker (Ravestein & Driedonks, 1986). Polypeptide A_1 occurs in all varieties described so far (Yamagishi *et al.*, 1983) and the solubility of acidic polypeptides is maintained after heating. With regard to both preparation of immunoreagent and samples for ELISA, different approaches have been taken to convert the protein to the same antigenic form. Hitchcock *et al.* (19 81) and Rittenburg *et al.* (1987) used an ELISA procedure which is specific for soy proteins that have been urea-denatured and then renatured. Ravestein & Driedonks (1986) demonstrated that the high solubilizing activity of the anionic detergent SDS allows complete recovery of soy proteins from meat samples. Furthermore, the antigenic status of SDS-denatured glycinin polypeptides A_1 and A_2 was independent of processing conditions.

Because the three-dimensional structure of proteins disappears on SDS-treatment, antibodies against SDS-denatured proteins will recognize predominantly continuous epitopes as they occur in the primary structure of the protein antigen. Therefore, it is claimed that the destruction of the native structure during food processing, e.g. heating or texturation, does not affect the immunoreactivity with antibodies against sequential epitopes of SDS-denatured proteins. Ideally, the SDS-treatment of different processed products containing native and/or various forms of denatured soy proteins brings all proteins to a common SDS-denatured conformation. This helps to normalize the response in the immunoassay to different types of samples, irrespective of processing conditions.

The procedure for antigen preparation presented in this paper provides a rapid method to obtain SDS-denatured proteins in high purity from SDS-PAGE. KDS-staining of SDS-protein complexes allows the visualization of soy proteins separated by SDS-PAGE without precipitation by organic solvents that are used with Coomassie protein staining. Hence, slices of polyacrylamide gel containing SDS-denatured proteins can be used directly for immunization where the acrylamide gel itself does not induce an immune response.

IgY antibodies obtained in this study are claimed to recognize a defined range of sequential epitopes of SDS-denatured glycinin A. It is suggested that the specific interaction with SDS-denatured glycinin A subunits is controlled by high affinity IgY antibodies against the SDS-denatured protein conformation and depends on the presence of SDS during incubation. A cross-reacting antigen generally has a lower affinity and thus the strength of antigen–antibody interaction is low relative to the strength of SDS binding. So the effect of low concentrations of the anionic detergent SDS on the elimination of cross-reactivity may be due to a covering-up of binding sites involved in low-affinity protein-IgY interactions. Accordingly, cross-reactivity of the phylogenetically related storage proteins from faba bean and pea (Fig. 4) was not obvious from the ELISA (SDS present) as contrasted with immunolocalization on blots (SDS absent). All non-soy proteins gave quite the same quantitative ELISA response (Table 1) corresponding to less than 0·5 μg ml^{-1} soy protein suggesting a negligible amount of unspecific reaction in the ELISA system. Consequently, the ELISA system can safely be applied at concentrations down to at least 1 μg ml^{-1} soy protein.

With respect to quantitation of soy protein in dairy-like products, it has to be considered that the ELISA system utilized here measures the concentration of acidic glycinin polypeptides and not the soy protein or soy drink concentration. Soy protein contains 40–50% glycinin which consists of acidic and basic subfractions (Brooks & Morr, 1985). However, there was a linear relationship between the known versus the measured values (Fig. 2, curve A), so that the slope can be used to calculate the conversion factor from glycinin A to soy protein and soy milk, respectively.

The ELISA technique using IgY antibodies against SDS-denatured soy glycinin A could be modified to detect soy proteins in different dairy-like products. Furthermore, the method for the preparation of IgY immunoreagents against SDS-treated proteins should be applicable to the analysis of other non-milk proteins. Studies are currently in progress to determine the usefulness of IgY antibodies against synthesized peptide fragments (single continuous epitopes) using peptide-carrier protein conjugates for immunization.

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