Investigation of the Relationship between *in Vitro* ELISA Measures of Immunoreactive Soy Globulins and *in Vivo* Effects of Soy Products

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Concentrations of glycinin and β-conglycinin were determined in 15 soy products using various enzyme-linked immunosorbent assay (ELISA) formats with specific polyclonal antisera (Pabs) and (or) monoclonal antibodies (Mabs). Nine of these products were evaluated *in vivo* for apparent digestibility of nitrogen and plasma anti-soy antibody titers in preruminant calves. In inhibition ELISAs, Mabs led to much higher values for antigen concentrations than Pabs. Compared to inhibition ELISAs with Pabs, two-site ELISAs associating Mabs and Pabs gave similar values for glycinin and higher values for β-conglycinin. However, *in vitro* tests which best predicted *in vivo* responses to soy were those using inhibition with Pabs, particularly against native β-conglycinin: with antigen concentrations correlation coefficients were negative for apparent digestibility of nitrogen and positive for anti-soy antibody titers. However, Mabs were more powerful tools for studying effects of treatments on soy immunoreactivity.

Keywords: *Calf nutrition; ELISA; protein; soy*

INTRODUCTION

Soy has become an important protein source as a cheap alternative to skim milk in formulas for young mammals. However, it contains a variety of antinutritional factors, including enzyme inhibitors and lectins which can be usually inactivated by thermal processing (Huisman, 1989). In addition, the storage globulins, glycinin and β-conglycinin, which represent two-thirds of the protein in the bean (Murphy and Resurreccion, 1984), are known to resist conventional treatments and digestion by mammalian proteases (Nielsen *et al.*, 1988; Sissons and Thurston, 1984). These proteins are also immunogenic in young calves and pigs and have been suspected to interfere with intestinal function via immunological mechanisms (Sissons, 1982; Lallès *et al.*, 1993b). Glycinin, also called 11S globulin, represents more than half of total globulins. It comprises a heterogeneous mixture of polypeptides involved in a hexameric structure ($M_r \approx 360\,000$) with a subunit molecular weight of around 60 000 (Guéguen and Azanza, 1985). In fact, this subunit associates an acidic polypeptide ($M_r \approx 40\,000$) and a basic polypeptide ($M_r \approx 20\,000$) disulfide bridged together. Known as 7S globulin, β-conglycinin consists of two acidic (α and α') and a basic (β) subunit of respective M_r's of 76 000, 72 000, and 53 000, which form trimers of M_r 150 000−200 000 (Guéguen and Azanza, 1985). This globulin accounts for 20−30% of the protein in the bean (Murphy and Resurreccion, 1984; Guéguen and Azanza, 1985).

Concentrations of antigens in soy ingredients for milk formulas were first evaluated semiquantitatively using a passive hemagglutination inhibition assay in which polyclonal antisera (Pabs) had been raised against native globulins (Kilshaw and Sissons, 1979). Passive hemagglutination inhibition was also successfully applied to monitor hot aqueous ethanol denaturation of soy protein (Sissons *et al.*, 1982a,b) and to demonstrate resistance of glycinin and β-conglycinin to digestion (Sissons and Thurston, 1984). Another semiquantitative test based on enzyme-linked immunosorbent assay (ELISA) inhibition, still using anti-soy globulin Pabs, was set up in the mid-1980s (Heppell *et al.*, 1987). Results were generally expressed in titers ($\log_2$ dilution), making it difficult to conduct interlaboratory comparisons. Finally, relationships between *in vitro* and *in vivo* data were scarce. For example, Tukur *et al.* (1993) showed that apparent digestibility of nitrogen at the ileum of calves was negatively correlated with ileal flow of glycinin, as assessed by a quantitative inhibition ELISA by Pabs.

Beside nutrition, soy protein is widely used in food technology to improve functional properties such as foaming, gelling, or emulsification during processing. However, links between protein structure and function are still obscure. Immunoassays employing monoclonal antibodies (Mabs) have been proposed to investigate the effects of processing on the structure of protease inhibitors of the Kunitz and Bowman−Birk families (Brandon *et al.*, 1987, 1988, 1989). More recently, a Mab (IFRN 0025) specific for acidic subunits of glycinin has become available (Carter *et al.*, 1992). In a direct ELISA, the reactivity of this Mab was shown to increase 10-fold after partial trypsinolysis, while Mab recognition virtually disappeared following more complete proteolysis (Plumb *et al.*, 1994). Heating glycinin to 80 °C had no effect on immunoreactivity, as assessed using a two-site ELISA format; further heating decreased epitope recognition by 50%, although immunoreactivity increased sharply above 92 °C (Plumb *et al.*, 1994). An anti-β-conglycinin Mab (IFRN 0089) was produced

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Table 1. Soy Globulin ELISAs Used for Analysis of Soy Products

soy globulin	antibody	ELISA format	specificity
glycinin	Pab LJR J4	inhibition	intact glycinin
	Mab IFRN 0025	inhibition	binds proteolytic intermediates and thermally denatured glycinin; epitope lies within acidic polypeptides
	Mab IFRN 0025 and Pab R103b$_3$	two-site	recognize proteolytic intermediates and thermally denatured glycinin
β-conglycinin	Pab LJR J2	inhibition	intact β-conglycinin
	Mab IFRN 0089	inhibition	recognizes epitopes in acidic regions of α and α' subunits of β-conglycinin
	Mab IFRN 0089 and Pab R195b$_3$	two-site	recognition of thermally denatured β-conglycinin is 3-fold greater than native

recently which is specific for the α,α' chains (Plumb *et al.*, 1995). Heating at temperatures above 80 °C increased β-conglycinin immunoreactivity markedly, as assessed by a two-site ELISA using this Mab (Plumb *et al.*, 1995).

The aim of the present study was to ascertain whether Mabs (which recognized protease-modified glycinin or native β-conglycinin) and Pabs (which recognized native or near-native molecules) employed in various ELISA formats were of use in comparing concentrations of antigens in soy products and hence to predicting *in vivo* responses to soy. A preliminary account of this work has been presented elsewhere (Lallès *et al.*, 1993a).

MATERIALS AND METHODS

Materials. *(1) Materials Used at INRA Rennes.* All soy (*Glycine max*) products were prepared industrially (Lallès *et al.*, 1995a,b; Toullec *et al.*, 1994; Tukur *et al.*, 1995), except two flours (DF 1, DF 2) which were defatted using petroleum spirit (bp 40−60 °C) at ambient temperature (20 °C) in this laboratory. Gelatin, Tween 20, anti-rabbit IgG-horseradish peroxidase conjugate, and substrate used for ELISA [2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)] were all purchased from Sigma Chemical Co., Saint-Quentin Fallavier, France. Microtiter plates were from NUNC Denmark. The Argus 300 plate reader was from Packard Instruments Co., Meriden, CT.

(2) Materials Used at IFR Norwich. Bovine serum albumin, anti-mouse IgG-horseradish peroxidase conjugate, and Tween 20 were all purchased from Sigma Chemical Co., Poole, Dorset, U.K. NUNC Immunoplate I microtitration plates were obtained from Gibco Europe, Paisley, U.K. Substrate for ELISA assays, based on 3,3′,5,5′-tetramethylbenzidine was supplied by Cambridge Veterinary Sciences, Cambridge, U.K. The Titertek Multiskan MCC plate reader and the Titertek Multiplate washer 120 were purchased from Flow Laboratories Ltd., Thame, U.K. All other reagents were of AR grade and were obtained from BDH Chemicals Ltd., Lutterworth, U.K., unless otherwise stated.

Soy Products and Purified Soy Globulins. Immunochemical tests were carried out on 15 soy products, including three raw defatted flours (DF), four defatted and toasted flours (TF), three water-extracted and toasted concentrates (WETC), two soy protein concentrates extracted using hot aqueous ethanol (AEHC), and three water-extracted and partially proteolyzed concentrates (WEPC). Products TF 4, WETC 3, and WEPC 2 had been subjected to additional treatments not disclosed by the manufacturers. All the soy products except DF preparations were used as protein sources in milk substitutes. However, three sets of *in vivo* data were discarded because two products (TF 3, AEHC 2) were involved in a study on ileal instead of fecal digestibility, and product WEPC 3 was a mixture of hydrolyzed soy protein (75% on a CP basis) and sweet whey plus synthetic amino acids (Lallès *et al.*, 1995b). The distributions of crude protein between peptides and native, aggregated, or carbohydrate-linked fractions were assessed according to Visser and Tolman (1993).

Glycinin and β-conglycinin used at INRA Rennes and IFR Norwich were prepared from raw defatted soy flour as described by Carter *et al.* (1992) and Tukur *et al.* (1993).

Immunochemical analyses at INRA Rennes were carried out after extraction of soy products in borate buffer (100 mM $NaBO_3$, 0.15 M NaCl, pH 8.0) for 1.5 h at room temperature (Tukur *et al.*, 1993). ELISA tests carried out at IFR Norwich using Mabs IFRN 0025 and IFRN 0089 were performed in samples extracted in Tris buffer (50 mM Tris-HCl, 0.2 M NaCl, pH 8.2) for 1.5 h at room temperature (Plumb *et al.*, 1994). These two protocols were found to extract essentially similar proportions of soluble protein (data not shown). The concentration of protein in solutions was determined using methods of Kjeldahl (crude protein CP = [N] × 6.25) or Lowry *et al.* (1951).

Antibody Preparations. Rabbit polyclonal antisera were produced against native glycinin and β-conglycinin (Tukur *et al.*, 1993). Rabbit Pab R103b$_3$ was produced against subtilisin-treated glycinin as described by Carter *et al.* (1992) and recognized both acidic and basic polypeptides of glycinin by immunoblotting (Plumb et al 1994). Rat Mab IFRN 0025 was produced against subtilisin-treated glycinin and was specific for its acidic polypeptides (Carter *et al.*, 1992). A rabbit Pab (R195b$_3$) was prepared against native β-conglycinin (Plumb *et al.*, 1995), and murine anti-β-conglycinin Mab IFRN 0089 recognized an epitope present only in the acidic α and α' subunits (Plumb *et al.*, 1995).

Enzyme-Linked Immunosorbent Assays (ELISAs). Various ELISA formats were set up (Table 1). Inhibition ELISAs with Pabs against native glycinin and β-conglycinin were used as described by Tukur *et al.* (1993), and with anti-glycinin Mab IFRN 0025 and anti-β-conglycinin Mab IFRN 0089 as described by Plumb *et al.* (1995). A two-site (or sandwich) ELISA was also used for glycinin analysis, which employed Mab IFRN 0025 as the capture antibody, and with rabbit Pabs R103b$_3$ as the detector antibody (Plumb *et al.*, 1994). In the two-site ELISA for β-conglycinin, Pabs R195b$_3$ served as the capture antibody and Mab IFRN 0089 as the detector antibody (Plumb *et al.*, 1995).

***In Vivo* Digestibility and Immunogenicity of Soy Products.** Four experiments were conducted in which nine soy ingredients for milk replacers were evaluated in groups of five to seven preruminant calves aged between 2 and 4 months (Lallès *et al.*, 1995a,b; Toullec *et al.*, 1994; Tukur *et al.*, 1995). Milk substitutes were formulated to contain 58−72% of the CP supplied by soy products. The remaining was provided by skim milk powder or whey plus synthetic amino acids. Concentrations of CP and fat were 20−22% and 18−20% on a dry matter basis, respectively. Milk replacers were fed daily at levels of 58−60 g of dry matter/kg of body weight$^{0.75}$. Apparent digestibilities of dietary nitrogen and soy nitrogen were determined at the fecal level as described previously (Lallès *et al.*, 1995a,b; Toullec *et al.*, 1994; Tukur *et al.*, 1995). In each study, body weight gain was calculated over the experimental periods and expressed as a proportion of values in control groups. Plasma anti-soy antibody titers were determined kinetically using passive hemagglutination (Kilshaw and Sissons, 1979). Anti-soy antibody titers retained for present calculations were those recorded on the last occasion in each trial and were corrected for background titers observed in control groups.

Statistical Analyses. Concentrations of immunoreactive glycinin and β-conglycinin in soy products were expressed on a CP basis. Owing to the non-Gaussian distribution of data, medians and ranges were considered. Immunoreactivity values between ELISA formats were compared using the

Table 2. Individual and Median Values (g/100 g of CP) for Immunoreactive Glycinin in Soy Products[a]

soy product	ELISA format[b] inhibition polyclonal	inhibition monoclonal	two-site monoclonal/polyclonal
defatted flours			
DF 1*	12.4	5.7	7
DF 2*	26.9	2.3	20.3
DF 3*	28.7	0	0.58
toasted flours			
TF 1	3.9	7.5	0.17
TF 2	2.7	34.6	0.53
TF 3*	1.7	29.3	0.26
TF 4	0.1	300	0.03
water-extracted and toasted concentrates			
WETC 1	2.0	30.2	0.18
WETC 2	3.3	40	0.37
WETC 3	0.003	94.4	0.02
alcohol-extracted and heated concentrate			
AEHC 1	0	262	0
AEHC 2*	0	77.4	0
water-extracted and partially proteolyzed concentrate			
WEPC 1	0	17.1	0
WEPC 2	1.1	417	0.13
WEPC 3*	0	1246	1.4
median[c] ($n = 15$)	1.7[a]	34.6[b]	0.18[a]
median[c] ($n = 9$)	1.1[a]	40[b]	0.13[a]

[a] Products noted with an asterisk were excluded from *in vivo* studies. [b] For details, see Table 1. [c] Medians with different superscript letters in the same row are different ($P < 0.05$).

nonparametric one-way analysis of variance of Friedman (Hollander and Wolfe, 1973). Linear correlation coefficients among ELISA tests and with *in vivo* data were determined using the rank test of Spearman (Dagniélie, 1970).

RESULTS

Six ELISA formats were used to investigate the immunoreactivity of glycinin (three tests) and β-conglycinin (three tests) in 15 soy products, out of which nine served as protein ingredients in milk substitutes and were further evaluated in calves. Median contents for total and soluble CP for the whole set of soy products were 58.5% of dry matter and 17% of CP, respectively. Medians were 66 and 15% for the subset of nine soy ingredients evaluated *in vivo*.

Glycinin Immunoreactivity. Median concentrations of immunoreactive glycinin ranged between 0.18 and 34.6 g/100 g of CP (Table 2). Inhibition ELISA with Mabs provided data significantly higher ($P < 0.05$) than those obtained by the other methods.

When the inhibition ELISA with Pabs was used, it was found that conventional toasting reduced the immunoreactivity of products by 5−10 times, water extraction per se having no additional effect on reactive glycinin concentrations. Products TF 4 and WETC 3 had been subjected to additional treatments not disclosed by the manufacturers; this was probably responsible for the great reduction in glycinin reactivity observed with this ELISA. By contrast, hot aqueous ethanol treatment and proteolysis removed soy glycinin reactivity completely for all products except WEPC 2. These results may reflect the degree of proteolysis in the samples.

Contrasting results were obtained using the anti-glycinin Mab, with differences between ELISA formats also being apparent (Table 2). Industrial defatting (DF 3) drastically reduced the reactivity to IFRN 0025 when used in the inhibition ELISA, whereas toasting (alone or with water or alcohol extraction) and proteolysis both increased immunoreactivity by up to as much as 200 times (WEPC 3) that observed for DF 1. Interestingly, this inhibition ELISA was able to discriminate between products of a given family of treatments. For example, Mab IFRN 0025 reacted more strongly with toasted flour TF 4 to which additional treatments had been applied, probably involving severe heating and (or) partial proteolysis. Similarly WEPC 3 may have been hydrolyzed differently to WEPC 1 and hence was more immunoreactive with IFRN 0025. Glycinin immunoreactivities determined by this Mab in a two-site ELISA were more often in line with those by inhibition ELISA using Pabs. This probably reflects the use of an anti-glycinin Pab as the detector antibody and the more complex kinetics of a two-site compared to an inhibition ELISA.

Comparing ELISA formats for glycinin, data from inhibition ELISA using Pabs were correlated negatively with those obtained by inhibition ELISA using Mabs (Spearman's correlation coefficient $r_s = -0.74$, $P < 0.01$) and positively with those obtained by two-site ELISA using Mabs ($r_s = 0.72$, $P < 0.01$). By contrast, correlation between inhibition ELISA with Mabs and two-site ELISA with Mabs were nonsignificant.

***β*-Conglycinin Immunoreactivity.** Both ELISAs using the anti-β-conglycinin Mab gave significantly higher ($P < 0.05$) median concentrations of immunoreactive β-conglycinin than inhibition ELISA with Pabs (Table 3).

Concentrations of β-conglycinin as determined by inhibition ELISA using Pabs varied according to treatments, as observed for glycinin in a similar ELISA format. However, β-conglycinin reactivity appeared to be more sensitive to partial proteolysis than glycinin with this assay. Again, contrasting observations were made for the ELISA inhibition with Mab IFRN 0089. Industrial defatting (DF 3) increased reactivity by 7-fold, while most other treatments tended to decrease the reactivity of this Mab to various degrees. When employed in a two-site ELISA, Mab IFRN 0089 behaved somewhat differently from inhibition ELISA, β-conglycinin immunoreactivity of laboratory defatted flour DF 1 being 3−4-fold higher when determined with the latter

Table 3. Individual and Median Values (g/100 g of CP) for Immunoreactive β-Conglycinin in Soy Products[a]

soy product	immunoassay format[b]		
	inhibition polyclonal	inhibition monoclonal	two-site polyclonal/monoclonal
defatted flours			
DF 1*	9	97	334
DF 2*	15.5	141	580
DF 3*	19.4	683	26.9
toasted flours			
TF 1	3.6	176	3.7
TF 2	1.3	10.4	39.8
TF 3*	2.9	16	0
TF 4	0	1.1	37.5
water-extracted and toasted concentrates			
WETC 1	2.5	43.7	0
WETC 2	1.5	61.5	88.7
WETC 3	0	0.57	436
alcohol-extracted and heated concentrates			
AEHC 1	0	4.3	47.6
AEHC 2*	0	0.85	163
water-extracted and partially proteolyzed concentrate			
WEPC 1	0	13.7	0
WEPC 2	0	66.7	363
WEPC 3*	0	162	0
median[c] ($n = 15$)	1.3^a	43.7^b	39.8^b
median[c] ($n = 9$)	0^a	13.7^b	39.8^b

[a] Products noted with an asterisk were excluded from *in vivo* studies. [b] For details, see Table 1. [c] Medians with different superscript letters in the same row are different ($P < 0.05$).

Table 4. Median Values and Ranges for *in Vivo* Data Obtained in Preruminant Calves[a]

item	median (range)
apparent digestibility of N in diets containing soy products (% control values)	88.3 (72.8−92.6)
calculated apparent digestibility of soy N (%)	76.0 (59.0−84.0)
body weight gain during the experimental periods (% control values)	83.3 (72.7−93.1)
corrected plasma anti-soy antibody titer (ASAT, $\log_2$ dilution)	2.2 (−0.1−7.9)

[a] Calculated from Lallès *et al.* (1995a,b), Toullec *et al.* (1994), and Tukur *et al.* (1995).

format. This immunoreactivity ratio was almost constant for four products under study (DF 2, TF 2, WETC 2, WEPC 2), strongly decreased for six products (DF 3, TF 1, TF 3, WETC 1, WEPC 1, WEPC 3), and increased to various degrees for four products (TF 4, WETC 3, AEHC 1, AEHC 2). Such variations in β-conglycinin immunoreactivity by two-site ELISA were not ascribed to a particular family of treatments.

Comparing ELISA formats for β-conglycinin, the only significant relationship was between inhibition ELISA using Pabs and inhibition ELISA using Mabs which gave results positively correlated with one another ($r_s = 0.67$, $P < 0.01$).

***In Vivo* Digestibility and Anti-Soy Antibody Titers.** Median values for apparent digestibilities of dietary and soy nitrogen, body weight gain, and anti-soy antibody titers were 88.3%, 76%, 83.3%, and 2.2 titer units, respectively (Table 4). Ranges of variation were large enough to provide significant ($P < 0.05 - < 0.001$) linear relationships with *in vitro* immunoreactivity of soy globulins.

Relationships between *in Vitro* and *in Vivo* Data. *(1) Glycinin.* Apparent digestibilities of dietary nitrogen and of soy nitrogen were negatively correlated, and anti-soy-antibody titers positively correlated, with glycinin immunoreactivity when it was determined using either inhibition ELISA with Pabs or two-site ELISA with Mabs ($P < 0.05 - < 0.01$; Table 5). By contrast, body weight gain was correlated with glycinin concentration only when inhibition ELISA with Mabs was used ($P < 0.05$). The strongest correlation was observed between apparent digestibility of dietary nitrogen and inhibition ELISA using glycinin Pabs ($r_s = -0.88$, $P < 0.01$).

(2) β-Conglycinin. Apparent digestibilities of dietary N and of soy N were negatively correlated ($P < 0.01 - < 0.001$), and anti-soy antibody titers positively correlated ($P < 0.01$), with β-conglycinin immunoreactivity only when it was determined by inhibition ELISA using Pabs (Table 5). The strongest correlation was observed between apparent digestibility of soy nitrogen and inhibition ELISA with β-conglycinin Pabs ($r_s = -0.90$, $P < 0.001$). *In vivo* data were not significantly correlated with *in vitro* data when the other ELISA formats were employed.

DISCUSSION

The present data clearly show that specific immunoreactivity may strongly depend on the type of antibodies (Pabs vs Mabs), the ELISA format (inhibition vs two-site), and finally the technological treatment applied to soy products. It is not so surprising because the antibodies used here were prepared against native or denatured molecules and presented particular binding patterns.

In the case of glycinin, hyperimmune sera Pab LJR J4 raised against the native molecule recognized acidic chains by immunoblotting (H. M. Tukur and J. P. Lallès, unpublished data). By contrast, Pabs R103b_3 raised against subtilisin-degraded glycinin recognized both the intact and the degraded molecule by ELISA, and it bound to acidic and basic chains by immunoblotting (Carter *et al.*, 1992; Plumb *et al.*, 1994). The monoclonal anti-glycinin antibody IFRN 0025 was prepared against subtilisin-treated glycinin; it recognized both native and degraded molecules by direct ELISA, and it bound to native and degraded forms of acidic polypeptides (S2, S3, S4) by immunoblotting (Carter *et al.*, 1992). Furthermore, studies on effects of heating and proteolysis on epitope−Mab binding patterns generated the idea

Table 5. Matrix of Spearman's Rank Test Correlation Coefficients between *in Vitro* and *in Vivo* Data

		in vivo parameters[a]			
protein	ELISA format	ADDN[b]	ADSN[c]	body weight gain	anti-soy antibody titer
glycinin	inhibition polyclonal	−0.88[b]	−0.75[a]	−0.59	0.82[b]
	inhibition monoclonal	0.45	0.59	0.75[a]	−0.65
	two-site	−0.79[a]	−0.67[a]	−0.47	0.74[a]
β-conglycinin	inhibition polyclonal	−0.89[b]	−0.90[c]	−0.66	0.89[b]
	inhibition monoclonal	−0.38	−0.27	−0.22	0.36
	two-site	0.26	0.43	0.13	−0.44

[a] [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$. [b] ADDN apparent digestibility of dietary nitrogen. [c] ADSN apparent digestibility of soy nitrogen.

that Mab IFRN 0025 recognized a continuous epitope, possibly repeated on acidic chains, which may be located near the surface but sterically obscured in the intact protein (Carter *et al.*, 1992; Plumb *et al.*, 1994). Transient decline in immunoreactivity with increasing temperatures could be due to either denaturation of the acidic chains or steric hindrance following protein aggregation (Plumb *et al.*, 1994).

In the case of β-conglycinin, our hyperimmune serum Pab LJR J2 recognized the acidic subunits (H. M. Tukur and J. P. Lallès, unpublished data). The epitope, or repeated epitopes, recognized by Mab IFRN 0089 was probably located in the N-terminal sequence of the α/α′ subunits, not present on the β subunit (Plumb *et al.*, 1995). When this Mab was used in inhibition ELISA, immunoreactivity increased as β-conglycinin was heated, reaching a maximum at 65 °C (Plumb *et al.*, 1995). This unusual thermal pattern corresponds to a partially hidden epitope, or multiple binding sites, located on disordered structures of α/α′ subunits of β-conglycinin (Plumb *et al.*, 1995).

The glycinin and β-conglycinin Pabs used here probably bound to discontinuous surface epitopes because thermal treatments strongly decreased immunoreactivity, and more drastic treatments including hot alcohol denaturation and proteolysis completely abolished it. Contrasting with this, glycinin immunoreactivity determined by inhibition ELISA with Mab IFRN 0025 most often increased to various degrees following thermal treatment, hot alcohol denaturation, and proteolysis. Although actual processing conditions for present products are not available, particular treatments, or succession of treatments, like those applied to TF 4, AEHC 1, WEPC 2, or WEPC 3, increased considerably (i.e., 50−200 times) glycinin recognition by Mab IFRN 0025 in inhibition ELISA. These observations are consistent with the behavior of this Mab whose epitope recognition is decreased at temperatures between 80 and 92 °C or following severe proteolysis and increased above 92 °C or following partial proteolysis to generate immunoreactive intermediates (Plumb *et al.*, 1994).

Thermal denaturation of pure β-conglycinin has been studied using Mab IFRN 0089 in a two-site ELISA (Plumb *et al.*, 1995). Antibody recognition increased with temperature, reaching a maximum at 65 °C for which immunoreactivity was 3-fold higher than unheated β-conglycinin; between 65 and 95 °C, it remained at least twice as high as with unheated protein (Plumb *et al.*, 1995). The influence of higher temperatures or proteolysis has not been studied yet. Here, Mab binding as assessed by inhibition ELISA increased 5−7-fold following industrial defatting. This involves desolvantation at 65 °C, a temperature that corresponds with thermal transition of the β-conglycinin molecule (Plumb *et al.*, 1995). Heat treatment reduced this immunoreactivity, in agreement with patterns of thermal denaturation of pure β-conglycinin, as mentioned above. This was so for hot alcohol denaturation and proteolysis. However, large variations in immunoreactivity observed among the present products would suggest industrial processing to be more severe, and probably epitope−Mab interactions to be of higher complexity, than laboratory studies with pure protein. This conclusion is further supported by the negative correlation ($r_s = -0.84$, $P < 0.01$) recorded between values of inhibition ELISA with Mabs and the proportion of aggregated protein.

Plumb *et al.* (1994) emphasized how critical the choice of ELISA format is in the monitoring of batch-to-batch variation in soy immunoreactivity. They observed that soy flours were not reactive using a direct ELISA with glycinin Mab IFRN 0025 and yet possessed up to 10 times the reactivity of the untreated flour when analyzed using a two-site ELISA. Present work extents these observations because data from inhibition ELISA and two-site ELISA with Mabs did not correlate with one another, irrespective of the soy globulin considered. Actual reasons for such discrepancies are unknown and await further investigation.

Semiquantitative immunological tests have been proposed to predict the suitability of soy ingredients in milk replacers for calves (Kilshaw and Sissons, 1979; Sissons *et al.*, 1982b) and allergic infants (Heppell *et al.*, 1987). Extending these observations, present results clearly indicate that concentrations of immunoreactive globulins, especially β-conglycinin, can accurately predict *in vivo* digestibility of soy nitrogen and immunogenicity, as far as immunoassays involving polyclonal sera raised against native proteins are concerned. This was independent of the type of immunoassay because highly significant relationships with *in vivo* data were also observed when these Pabs were introduced into quantitative passive hemagglutination inhibition and dot blotting assays (H. M. Tukur and J. P. Lallès, unpublished data). Apart from the two-site ELISA employing anti-glycinin Mab IFRN 0025 and Pab R103b3, results generated by Mabs in ELISAs contrasted sharply with those obtained by Pabs formats and did not correlate with *in vivo* data. This could be expected since a given Mab recognizes only one epitope. Although the spatial organization of the Mab epitopes is sensitive to thermal processing or proteolysis (Plumb *et al.*, 1994, 1995), such epitopes may not be representative of the overall behavior of soy protein *in vivo*, as far as specific antibody titers in plasma and apparent digestibility of nitrogen are concerned.

Whether relationships between fecal digestibility of nitrogen and *in vitro* immunoreactivity of soy products are causal is unclear. One the one hand, a negative correlation between ileal digestibility of nitrogen and ileal flow of immunoreactive glycinin was observed in calves consuming the antigenic soy flour TF 3 for a short period of time (Tukur *et al.*, 1993); by contrast, ileal flow of β-conglycinin was 10 times lower than that of glycinin

and did not correlate with ileal digestibility of nitrogen. On the other hand, direct involvement of soy globulins as allergens has been suspected for some time (Kilshaw and Sissons, 1979). But it is only recently that glycinin and β-conglycinin were shown to induce immediate skin reactions; in addition, β-conglycinin, but not glycinin, has been found to stimulate both delayed skin thickening and *in vitro* lymphoproliferation (Lallès *et al.*, 1996). Therefore, immune-mediated effects of soy globulins on gut function probably participate in enhancing losses of endogenous protein and undigested dietary protein at the end the small intestine. Such a premise is supported by the fact that β-conglycinin is the best predictor of soy protein digestibility in this study, and the strongest immune stimulator in soy (Lallès *et al.*, 1996).

CONCLUSION

Relationships between concentrations of glycinin and β-conglycinin in soy products and nitrogen digestibility or immunogenicity in the calf can be revealed with ELISA employing hyperimmune sera produced against native globulins. Monoclonal antibodies are powerful tools for studying conformational changes of molecules induced by technological treatments, but the results strongly depend on the ELISA format employed. The Mabs used here presented a low predictive value for *in vivo* parameters, most probably because the concerned epitopes were not representative enough of the overall susceptibility of soy globulins to digestion. For Mabs to be employed effectively in such prediction would require antibodies specific for allergenic epitopes. The Mabs could also be of use in determining the presence of denatured soy products in milk replacers. Both polyclonal and monoclonal antibody reagents are currently used to identify and characterize soy immunoreactive polypeptides which escape digestion in the calf.

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