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J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/jf5052128 • Publication Date (Web): 11 Feb 2015

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1 **Rapid and Sensitive Detection of the Food Allergen Glycinin**
2 **in Powdered Milk Using a Lateral Flow Colloidal Gold**
3 **Immunoassay Strip Test**

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ABSTRACT: A rapid immunochromatographic lateral flow test strip in a sandwich format was developed with the colloidal gold-labeled mouse anti-glycinin monoclonal antibody (mAb) and rabbit anti-glycinin polyclonal antibody (pAb) to specifically identify glycinin, a soybean allergen. The test strip is composed of a sample pad, a conjugate reagent pad, an absorbent pad and a test membrane containing a control line and a test line. This test strip has high sensitivity, and results can be obtained within 10 min without sophisticated procedures. The limit of detection (LOD) of the test strip was calculated to be 0.69 mg/kg using an optical density scanner that measures relative optical density. The assay showed high specificity for glycinin, with no cross-reactions with other soybean proteins or other food allergens. The recoveries of the lateral flow test strip in detecting glycinin in powdered milk samples ranged between 80.5 and 89.9% with relative standard deviations of less than 5.29% (intra-assay) and 6.72% (inter-assay). Therefore, the test strip is useful as a quantitative, semi-quantitative or qualitative detection method for glycinin in powdered milk. In addition, the test strip can be used to detect glycinin in other processed foods and may be a valuable tool in identifying effective approaches for reducing the impact of glycinin.

KEYWORDS: glycinin, immunochromatographic strip, colloidal gold, immunoassay

INTRODUCTION

The soybean is consumed extensively worldwide due to its nutritional and health benefits.¹ In addition to traditional powdered milk prepared exclusively from milk, other powdered milk products containing soybean protein have been developed. The soybean is an inexpensive source of protein compared with milk proteins.² Thus, with its ability to significantly reduce the prices of powdered milk, soybean protein is an attractive alternative to milk protein in powdered milk. However, the soybean is one of the “big eight” food allergens, and its undeclared addition to food presents a potential risk for allergic individuals.³ Approximately 0.4% of children are allergic to soybeans⁴. Feeding allergic children diets containing soybean proteins can cause a variety of symptoms involving the skin, gastrointestinal tract, and respiratory tract.⁵ According to the EU directive 2007/68/EC,⁶ food products containing soybeans and products thereof must be appropriately labeled, regardless of the amount added as an ingredient, to protect allergic consumers. Consequently, reliable quantification methods for detecting soybean allergens are necessary to ensure compliance with food labeling requirements and to avoid potential fraud associated with the addition of soybean protein to powdered milk.

Glycinin is the predominant soybean seed storage protein, accounting for approximately 50% of total soybean proteins,⁷ belongs to the 11S globulin family, and has been demonstrated to be one of the most predominant soybean allergens.⁸ Glycinin causes damage to intestinal morphology, immune function disorders, growth depression and diarrhea.⁹ As one of the major allergens found in the soybean, glycinin

58 is a good target protein for the specific detection of soybean protein. Thus, a sensitive
59 detection method is needed to protect allergic individuals from exposure to hidden
60 soybean allergens. Although quantification of soybean proteins can be performed by
61 high-performance liquid chromatography,¹⁰⁻¹² the method requires time-consuming
62 cleanup steps and expensive equipment. Other methods for the detection of allergens,
63 such as polymerase chain reaction (PCR) based on the amplification of specific DNA
64 fragments, have also been recently developed,¹³ but these methods are also
65 time-consuming and expensive. Many research groups have attempted to develop
66 methods for the detection of allergens using relatively inexpensive equipment and
67 high-throughput screening. The majority of the developed methods are based on
68 enzyme-linked immunosorbent assay (ELISA) for detecting soybean allergens. These
69 ELISA methods include competitive and sandwich formats, and have enabled rapid
70 qualitative and quantitative detection of soy allergens in complex foods.¹⁴ Tukur et
71 al.¹⁵ and Ma et al.¹⁶ developed competitive ELISA to determine concentrations of
72 glycinin. The competitive ELISA developed by Ma et al. was based on mAb, and
73 showed a working range of 0.3-11.2ng/mL. The sandwich ELISA format is more
74 powerful for the detection of proteins, in which the analyte to be measured is bound
75 between the capture antibody and the detection antibody.¹⁷ By using a mouse mAb as
76 a capture antibody and a rabbit pAb as the detected antibody, Chen et al. established a
77 sandwich ELISA for detecting glycinin that showed high specificity for glycinin with
78 a wider working range of 3-200 ng/mL and a LOD of 1.63 ng/mL.¹⁸

79 Recently, as a developed technique based on ELISA method,

immunochromatographic lateral flow test strips have been considered because they are inexpensive, rapid, portable and extremely simple to operate. Immunochromatographic lateral flow test strips in the sandwich format have become a popular diagnostic tool for detecting high-molecular-mass analytes, such as viruses, bacteria, hormones, and parasite antigens.¹⁹ However, these strips are not extensively used for the detection of food allergens, and there are no available lateral flow test strips for detecting glycinin. Therefore, in this study, we generated a pAb and a highly specific mAb against glycinin and developed a novel sandwich format lateral flow assay for the detection of glycinin in powdered milk.

89

90 MATERIALS AND METHODS

91 **Materials and Apparatus.** The following materials were used in this study:
92 soybean β -conglycinin, agglutinin, trypsin inhibitor, peanut agglutinin (PNA),
93 ovalbumin (OVA), casein, Freund's complete adjuvant (FCA), Freund's incomplete
94 adjuvant (FIA), 3,3',5,5'-tetramethylbenzidine (TMB) and mouse monoclonal antibody
95 isotyping reagents, which were purchased from Sigma-Aldrich (St. Louis, MO, USA);
96 PEG1500 was purchased from Roche (Mannheim, Germany); ELISA plates (96 wells)
97 and other cell culture plastic wares were obtained from Costar (Cambridge, MA); goat
98 anti-rabbit and goat anti-mouse IgG-horseradish peroxidase (HRP) were obtained
99 from Abbkine (Redlands, CA); fetal bovine serum (FBS) was obtained from Hyclone
100 (Logan, UT); Dulbecco's modified Eagle's medium (DMEM) was obtained from
101 BRL-Gibco (Grand Island, NY); RPMI-1640, HAT, and HT medium were purchased

102 from Invitrogen; nitrocellulose membrane, glass fiber, and absorbent pad were
103 purchased from Millipore; and eight-week old female BALB/c mice and New Zealand
104 White rabbits were obtained from the Laboratory Animal Center (Zhengzhou
105 University, China) and raised according to principles of the Henan Academy of
106 Agricultural Sciences Animal Care and Use Committee. All other reagents and
107 solvents were of analytical grade or higher.

108 The Model 450 Microplate Reader, electrophoresis cell vertical system and
109 semi-dry transfer cell used in this study were obtained from Bio-Rad (Richmond, CA).
110 All water used was produced by a Milli-Q Academic Water Purification System
111 (Millipore, Bedford, MA). The XYZ Biostrip Dispenser, CM 4000 Cutter, and
112 TSR3000 membrane strip reader employed were purchased from Bio-Dot. The
113 Multifuge X1R high-speed refrigerated centrifuge was purchased from Thermo
114 Scientific (Osterode, Germany), and the FreeZone 2.5 Liter Benchtop Freeze Dry
115 System was purchased from LABCONCO (Kansas City, MO).

116 **Glycinin Purification and Sample Protein Extraction.** Soybeans and powdered
117 milk were purchased at local supermarkets in Zhengzhou, China. Soybeans were
118 ground to a fine powder and passed through a 60 mesh sieve. The soybean powder
119 was then defatted according to the method of Hei et al.¹⁷ Soybean protein was then
120 extracted by making a slurry with a 15-fold volume of water (adjusted to pH 9.0 with
121 2 N NaOH) under continuous agitation for 1 h, followed by centrifugation at 9,000 g
122 for 30 min; the supernatant was then filtered through a 0.45 μ m Millex GP filter
123 (Millipore, Cork, Ireland). In accordance with a previous report²⁰, 0.01 M NaHSO₃

124 was added to the soybean protein supernatant, and the pH was adjusted to 6.4 with 2
125 N HCl. The mixture was kept on ice overnight, and the precipitate was collected by
126 refrigerated centrifugation at 6,500 g for 30 min at 4 °C. Crude extracts of glycinin
127 were purified using Sepharose CL-6B (Sigma-Aldrich, St. Louis, MO), and the
128 purified extracts were kept sealed after freeze drying. The purity of the glycinin was
129 estimated by HPLC according to the method of García et al.¹² A Waters HPLC system
130 (Milford, MA) equipped with a 600 Controller, In-Line Degasser AF, 2767 Sample
131 Manager, and 2996 Photodiode Array Detector was used. The mobile phases
132 employed were prepared with 0.1% (v/v) TFA in water, (mobile phase A) and 0.1%
133 (v/v) TFA in ACN (mobile phase B). Separation was performed with an RP perfusion
134 column (POROS R2/H; 100×4.6 mm id) packed with crosslinked
135 polystyrenedivinylbenzene beads (20 µm) from Perseptive Biosystems (Framingham,
136 MA). The chromatographic method consisted of a linear binary gradient from 5 to
137 60% B over 5 min. The flow rate was 5 mL/min, the injection volume was 500 µL,
138 and the detection was performed at 254 nm.

139 We procured commercially powdered milk without soybean included in the list of
140 ingredients. Powdered milk was used in this study to evaluate glycinin recovery by
141 the strip assay. The powdered milk was adulterated with different amounts of purified
142 glycinin before being subjected to the defatting and protein extraction procedures
143 described previously. The protein supernatants were analyzed either directly or
144 following further dilution as required by the strip test.

145 **Production of mAb Against Glycinin.** Three 8-week-old BALB/c female mice

146 were immunized with purified glycinin. The first dose consisted of 50 µg of
147 immunogen for subcutaneous injection as an emulsion of PBS and FCA. Three
148 subsequent injections were administered at 3-week intervals with the same dosage of
149 immunogen emulsified in FIA. Antisera samples were collected 4 weeks after the
150 fourth immunization, and the samples were screened for anti-glycinin activity by
151 ELISA. The mouse with the highest anti-glycinin activity received a fifth injection
152 intraperitoneally. Three days later, the spleen of the injected mouse was removed for
153 hybridoma production.

154 Hybridomas secreting anti-glycinin antibodies were generated by standard
155 methods²¹. Briefly, the spleen of the immunized mouse was removed, and the
156 splenocytes were isolated and fused with NS0 cells using PEG1500. The fused cells
157 were then distributed into 96-well culture plates in which mouse peritoneal
158 macrophages were prepared on the day before fusion and were grown with the
159 selective HAT medium. Ten days after fusion, the supernatants of hybridoma colonies
160 were recovered and screened by indirect ELISA for the secretion of mAb binding to
161 glycinin. Selected clones were subcloned by limiting dilution. Ascites fluids were
162 produced in paraffin-primed BALB/c mice, and the mAb was then purified from the
163 antiserum by ammonium sulfate precipitation. The subclass of the isotypes of the
164 mAb was determined using mouse monoclonal antibody isotyping reagents. The
165 measurement of monoclonal antibody affinity (K_a) was performed according to the
166 procedure described by Batty et al.²² The following formula was used to calculate K_a :

167
$$K_a = (n-1)/2(n[Ab']_t - [Ab]_t)$$

$$n=[Ag]t/[Ag']t$$

where [Ag]t and [Ag']t represent two different concentrations of coating antigen and [Ab]t and [Ab']t represent the corresponding concentrations of mAb.

Indirect ELISA and Competitive Indirect ELISA. The antibody titer was monitored by indirect ELISA. Microplates were coated with 100 μ L/well of purified glycinin (10 μ g/mL) in 0.05 M carbonate-bicarbonate buffer (pH 9.6) and then incubated at 37 $^{\circ}$ C for 2 h. After washing with PBST [PBS containing 0.05% Tween 20 (v/v), pH 7.4], the wells were blocked with 200 μ L of 5% skim milk in PBST and then incubated at 37 $^{\circ}$ C for 1 h. After another washing step, 100 μ L/well of purified glycinin with 2-fold serially diluted antibody in blocking buffer was added and incubated for 15 min at 37 $^{\circ}$ C. Following the washing step, goat anti-mouse IgG-HRP conjugate (1:5000 in blocking buffer, 100 μ L/well) was added and incubated for 30 min at 37 $^{\circ}$ C. The plates were washed again, and 100 μ L/well of TMB in chromogen buffer was added; color development was terminated after 10 min with 2 mol/L H_2SO_4 (100 μ L/well). The optical densities (ODs) were measured at 450 nm with a microplate reader. A competitive indirect ELISA was carried out to screen the antibody for anti-glycinin activity. Microplates were coated as described previously, and 100 μ L/well of glycinin serially diluted in PBS was then added with 100 μ L/well of antibody and incubated for 15 min at 37 $^{\circ}$ C. The following procedures were identical to those employed in indirect ELISA.

SDS-PAGE and Western Blot. The purified glycinin and isolated soybean protein were dissolved in PBS and mixed with SDS sample buffer, and the samples were then

190 loaded onto 5-12% polyacrylamide gradient gels. SDS-PAGE was performed at 90 V
191 until the tracking dye migrated to the bottom edge of the gel. The gel was stained with
192 0.25% Coomassie Brilliant Blue R250.

193 The SDS-PAGE gels were transferred onto nitrocellulose membranes for 1.5 h at 15
194 V. The membranes were blocked with 5% skim milk in PBST at 4 °C overnight, and
195 the membrane was incubated with anti-glycinin mAb (1:5000) at room temperature
196 for 1 h. After three PBST washes, the membrane was incubated with secondary
197 antibodies (horseradish peroxidase-conjugated goat anti-mouse IgG 1:5000) at room
198 temperature for 1 h, and 3-amino-9-ethylcarbazole (AEC) was used as a staining
199 substrate.

200 **Production of pAb Against Glycinin.** Two New Zealand white rabbits were used
201 to produce pAb. Both rabbits were immunized subcutaneously with 200 µg of purified
202 glycinin. The first subcutaneous injection was composed of an emulsion of PBS and
203 FCA. Three subsequent injections were administered at 3-week intervals with the
204 same dosage of immunogen emulsified in FIA. Four weeks after the fourth
205 immunization, blood samples were collected directly from the heart and were
206 screened for anti-glycinin activity by ELISA. The antibody was purified from the
207 antiserum by ammonium sulfate precipitation.

208 **Preparation of Colloidal Gold-labeled mAb.** Colloidal gold with a mean diameter
209 of 15 nm was produced by reduction of gold chloride with 1% sodium citrate (w/v), as
210 described previously²³. When preparing the colloidal gold-labeled mAb, the colloidal
211 gold solution was adjusted to pH 9.0 with 0.2 mol/L sodium carbonate. The optimum

ratio of mAb solution (1 mg/mL) to colloidal gold solution was determined according to a previous report²⁴. Approximately 100 μ L of mAb solution was added to 10 mL of the colloidal gold solution with constant stirring for 30 min. Then, 1 mL of 0.02 M sodium borate (pH 9.0) containing 10% BSA was added with stirring for 10 min to stabilize the conjugate. The colloidal gold-labeled antibody was washed three times with wash buffer (0.02 M sodium borate, pH 9.0; 1% BSA; and 0.1% sodium azide) by repeated centrifugation at 25,000 g and 10 °C for 30 min. The precipitate was re-suspended in 1 mL of wash buffer and stored at 4 °C before use.

Fabrication of the Colloidal Gold Immunoassay Strip. The conjugation pad (7 mm $\times$ 300 mm) was prepared by dispensing a desired volume of colloidal gold-labeled mAb onto a glass fiber pad (Millipore) using an XYZ Biostrip Dispenser, followed by drying at 56 °C for 1 h before storage at 4 °C. Sample and absorbent pads were made from nonwoven material (Millipore). The sample pad (15 mm $\times$ 300 mm) was saturated with a buffer (pH 8.0) containing 20 mmol/L sodium borate, 2% (w/v) sucrose, 2% (w/v) BSA, and 0.1% (w/v) NaN₃, and the pad was then dried and stored as described previously. The nitrocellulose membrane (20 mm $\times$ 300 mm) was spotted using the same dispenser, with the optimal pAb and anti-mouse IgG antibodies applied in the test and control lines, leaving a 0.5 cm space between the two lines. After drying for 1 h at 40 °C, the membrane was blocked with 2% (w/v) BSA and then dried, sealed, and stored under dry conditions. The absorbent pad was cut to dimensions of 20 mm $\times$ 300 mm. The sample pad, conjugate pad, blotted membrane, and absorption pad were assembled on a plastic backing support board (40 mm $\times$ 300

mm) sequentially with a 1-2 mm overlap. The master card was cut into 3 mm wide strips using a CM 4000 Cutter (Bio-Dot Irvine, CA, USA). The strips were then sealed in a plastic bag with desiccant gel and stored at 4 °C.

Test Procedure and Principle. In the detection test, 100 µL of standard solution or sample extract is allowed to react with colloidal gold-labeled mAb. The mixture then moves upward on the nitrocellulose membrane chromatographically via capillary action. For a positive sample, glycinin binds to the mAb, forming a gold/antigen/antibody complex, which binds to pAb and forms a red color band in the test region. Higher amounts of glycinin in the sample result in a stronger test line color. The absence of this band suggests a negative result. To serve as a procedural control, a red band will always appear in the control region regardless of the presence of glycinin.

If both the test and control lines turn red, the sample is recorded as positive, indicating the presence of glycinin. If the control line but not the test line is colored, the sample is considered negative. If only the red test line appears, the strip is considered invalid, and the test should be repeated using a new strip.

Evaluation of the Lateral Flow Assay. The sensitivity of the test strips was determined by testing a series of diluted glycinin standard sample extracts in which various concentrations of purified glycinin, i.e., 0, 50, 100, 200, 400, 800, 1600, 3200, 6400, and 12800 ng/mL, were used. Test lines were scanned with a Bio-Dot TSR3000 Membrane Strip Reader (BioDot, CA, USA). The assays were performed in triplicate using the test strip. The standard curve was constructed by plotting the

256 G/D×Area-relative optical density (ROD) or G/peak-ROD values obtained from the
257 standard samples against the logarithmic concentrations. The linearity of the analytes
258 was assessed by the coefficient of determination (R^2), and the LOD was calculated
259 from the regression equation.

260 To identify the specificity of the test strip, the soybean allergens (β -conglycinin,
261 agglutinin, and trypsin inhibitor) and other food allergens (PNA, OVA, and casein)
262 were tested at a concentration of 20 μ g/mL.

263 To determine the accuracy of the test strip, powdered milk samples were spiked
264 with purified glycinin at 3, 9, and 27 mg/kg, 100 g samples were defatted and mixed
265 with 1500 mL water for protein extraction procedures and then tested by the strip. The
266 optical density of the test line was measured using the test strip reader, and sample
267 values were calculated from the standard curve. Intra-assay precision was estimated
268 using one batch of the test strips for replication analysis. For inter-assay precision,
269 three batches of the test strips were used to detect the given samples. Precision was
270 expressed as the coefficient of variation (CV, %).

271

272 RESULTS

273 **Establishment of Hybridomas.** The purity of glycinin was estimated to be 86.5%
274 according to the HPLC analysis, which was sufficient for the immunization
275 experiments. The BALB/c mouse immunized with purified glycinin with the highest
276 titer (1:51200) was selected for subsequent experiments for monoclonal antibody
277 preparation.

278 Spleen cells from the mice immunized with glycinin were fused with NS0 myeloma
279 cells, and the resulting hybridomas were selected in HAT medium. One or more
280 growing hybridomas were observed in almost all wells after 7 days. The supernatants
281 from each well were screened for antibodies against glycinin by direct ELISA using
282 microtiter plates coated with glycinin. The cells from the wells showing the strongest
283 response ($OD_{450nm} > 2.0$) were tested again by competitive indirect ELISA for their
284 ability to recognize free glycinin. Six selected hybridomas were screened and further
285 cloned by limiting dilution. After culture and further screening, the most sensitive
286 hybridomas (i.e., 2C3H5) were intraperitoneally injected into mice to produce ascites
287 fluid, and the mAb was purified from the ascites fluid by ammonium sulfate
288 precipitation.

289 **Characterization of Antibody.** The pAb from rabbit No. 2 was selected for further
290 study due to its high titer of $1:1.024 \times 10^6$, which was the highest titer among the pAbs
291 produced. The mAb 2C3H5 titer was $1:5.12 \times 10^5$, and the affinity constant (K_a) was
292 4.27×10^9 L/mol. The subtype of the mAb was identified as IgG1.

293 Western blot analysis was conducted to determine the polypeptide chains of
294 glycinin that react with the mAb. Glycinin together with total soybean protein isolate
295 was reacted with mAb 2C3H5. The results showed that there was a single band with a
296 molecular weight of approximately 40 kDa, corresponding to the A3 acid polypeptide
297 chain of glycinin (Figure 1A). The Western blot results indicated that the mAb reacted
298 with the A3 acid polypeptide chain of glycinin specifically without cross-reaction with
299 other polypeptide chains of glycinin or other soybean proteins.

300 **Sensitivity of the Test Strip.** The sensitivity of the test strip was determined by
301 testing the series of diluted glycinin standard sample extracts. Test lines were scanned
302 with a Bio-Dot TSR3000 Membrane Strip Reader. The G/D×Area-ROD and
303 G/Peak-ROD values increased as the glycinin concentration in the samples increased,
304 but they were saturated above 3200 ng/mL (Figure 2 and Table 1).

305 The G/D×Area and G/Peak values of the ROD increased as the glycinin
306 concentration in the samples increased. The concentration of standard glycinin and the
307 G/D×Area ROD produced a sigmoidal dose-response curve, which showed good
308 linearity over the range of 50-3200 ng/mL. The standard curve was constructed by
309 plotting the G/D×Area-ROD percentage obtained from the standard samples against
310 the logarithm concentrations (Figure 3). The LOD with the scanner was quantitatively
311 defined as the amount of glycinin in the standard sample solution that caused a 10%
312 increase in the G/D×Area-ROD produced by the 0 ng/mL glycinin sample. Thus, a
313 glycinin concentration of 46.1 ng/mL (equivalent to 0.69 mg/kg) was calculated to be
314 the LOD for the test strip.

315 **Specificity of the Test Strip.** OVA, casein, PNA, soybean β -conglycinin, agglutinin
316 and trypsin inhibitor, each at a concentration of 20 μ g/mL, were tested, and the color
317 of the test line was the same as that of the negative control sample. The
318 cross-reactivity results demonstrated that the test strip for glycinin was highly specific
319 and showed negligible cross-reactivity with other food allergens (Figure 4).

320 **Recovery of Glycinin in Powdered Milk Sample.** To determine the accuracy of
321 the test strips, powdered milk samples containing 3, 9, and 27 mg/kg of glycinin were

322 tested. The test was performed in triplicate with a single batch of test strips. The
323 optical density of the test line was measured using the test strip reader, and sample
324 values were calculated from the standard curve. For intra-assay reproducibility, the
325 recoveries ranged from 82.3 to 89.9%, with the highest coefficient of variation
326 occurring at 5.29% (Table 2). For inter-assay reproducibility, three different batches of
327 the test strips were used for triplicate measurements of the samples. Recoveries
328 ranged from 80.5 to 87.7%, with the highest coefficient of variation occurring at
329 6.72%.

330

331 **DISCUSSION**

332 Food allergies have become an important health problem in developed countries,
333 affecting more than 1-2% of the population.²⁵ Undeclared allergens as contaminants in
334 food products pose a major risk for sensitized persons. These issues are important
335 because they could potentially have grave consequences for consumers and should be
336 addressed by establishing reliable standards and methods for risk assessment.

337 Soybean allergy is of particular importance because soybeans are widely used in
338 processed foods and therefore represent a particularly insidious source of hidden
339 allergens.²⁶ To date, many ELISA methods have been developed for the detection of
340 allergens using relatively inexpensive equipment and high-throughput screening.
341 However, 2 to 3 h are required to perform such assays. In contrast, the
342 immunochromatographic lateral flow test strip is a one-step assay that requires less
343 professional personnel for the rapid identification of various analytes.

344 The key requirement for a successful immunochromatographic lateral flow test is
345 the availability of antibodies with high specificity and the desired affinity.²⁷ In the
346 present study, the monoclonal antibody prepared by the selected clone, 2C3H5,
347 showed particularly high affinity for glycinin. The isotype of the mAb was identified
348 as IgG1. The mAb 2C3H5 showed no cross-reactivity with other soybean allergens
349 (soybean β -conglycinin, trypsin inhibitor and agglutinin), resulting in a high
350 specificity for glycinin.

351 Although some lateral flow assays designed for the determination of food allergens
352 have been reported,²⁸⁻³⁰ there has been no scientific report or sufficient validation of a
353 lateral flow assay for detecting glycinin. In the present study, a novel
354 immunochromatographic lateral flow test strip was developed using the sandwich
355 format, which is applicable for target analytes with more than one epitope (high
356 molecular mass analytes).³¹ To evaluate the validity and reliability of the lateral flow
357 test strip, sensitivity, specificity and recovery studies were performed for the
358 determination of glycinin in powdered milk. The results indicated that the lateral flow
359 test strip was sensitive and reliable, and the lateral flow assay showed no reactivity
360 toward other soybean allergens (β -conglycinin, agglutinin and trypsin inhibitor) or
361 other food allergens (PNA, OVA and casein).

362 In conclusion, a high-affinity and specific mAb against glycinin was produced by
363 immunizing mice with purified glycinin. Using the 2C3H5 mAb and rabbit pAb to
364 develop the lateral flow test strip resulted in a highly sensitive test with a LOD of 0.69
365 mg/kg for glycinin, which is sufficient for the determination of glycinin in powdered

366 milk. In addition, this lateral flow test strip can also be used to detect glycinin in other
367 processed foods (e.g., cheese and sausage) when samples are appropriately extracted.
368 Moreover, this effective method for detecting glycinin may be a valuable tool in
369 identifying effective approaches for reducing the impact of glycinin.

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375 **Founding**

376 This research was supported by the Basic and Cutting-edge Project of Henan Province
377 (132300413222) and the National Key Technology R&D Program of “12th
378 Five-Year Plan” (2014BAD13B05).

379 **Notes**

380 The authors declare no competing financial interest.

381

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474

475 **Tables and Figures**

476 **Table 1.** G/D×Area and G/Peak of the Relative Optical Density (ROD) of the Test
477 Lines of the Sample Extracts Added with Glycinin Standard Solutions.^a

Glycinin concentration (ng/mL)	G/D×Area-ROD (pixel)	G/Peak-ROD (pixel)
0	2.2518	0.0024
50	19.7731	0.0142
100	38.3656	0.0225
200	68.1175	0.042
400	93.8202	0.0666
800	128.8885	0.0927
1600	153.235	0.1055
3200	169.3604	0.1166
6400	169.0685	0.1163
12800	168.976	0.1147

478 ^a Sample extracts added to glycinin standard solutions were tested using the test strips,
479 and the test lines were scanned with a TSR3000 Membrane Strip Reader.
480 G/D×Area-ROD: mean density value of the sampled line points multiplied by the area
481 of the sampling window on the image. G/Peak-ROD: peak value of the ROD points of
482 the scanned line.

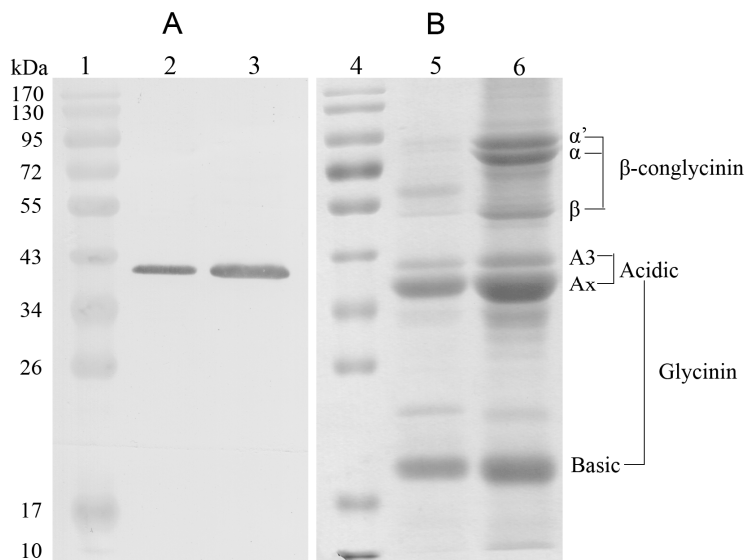
483

484

Table 2. Recovery and Intra- and Inter-assay Precision of the Test Strips for
Glycinin-Spiked Powdered Milk Samples.

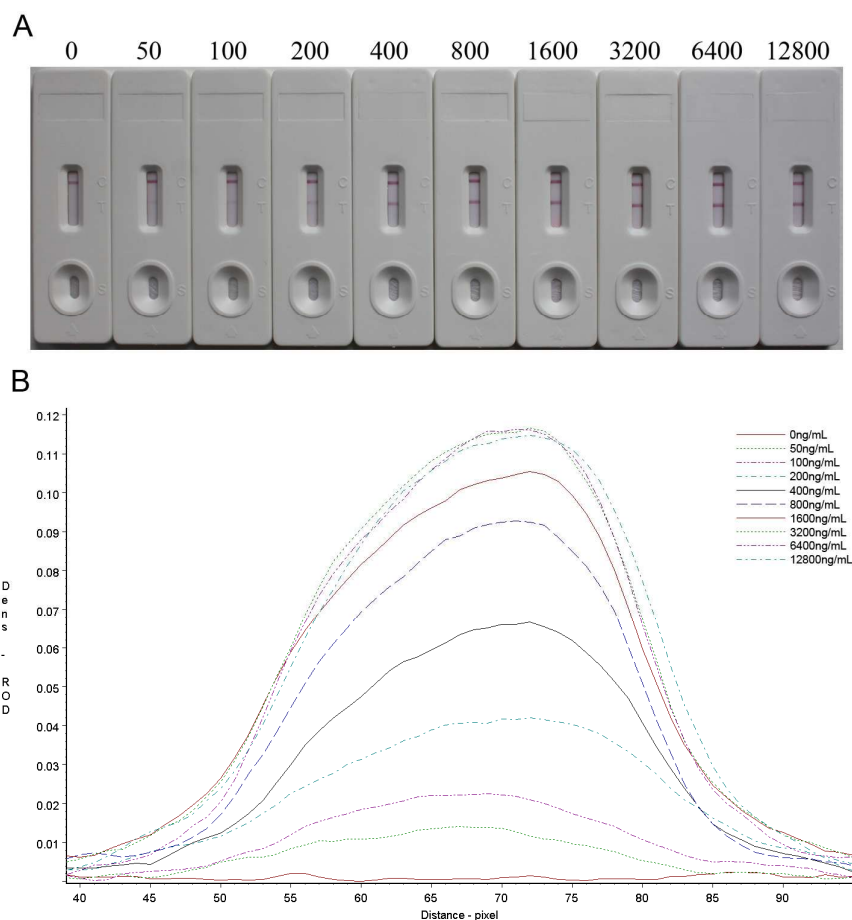
Spiked Glycinin (mg/kg)	Intra-assay			Inter-assay		
	mean±SD (mg/kg)	Mean recovery (%)	CV (%)	mean±SD (mg/kg)	Mean recovery (%)	CV (%)
3	2.47±0.13	82.3	5.24	2.42±0.16	80.5	6.72
9	7.59±0.37	84.3	4.85	7.53±0.48	83.6	6.38
27	24.26±1.28	89.9	5.29	23.67±1.55	87.7	6.56

Intra-assay precision was estimated using one batch of the test strips ($n=6$). For inter-assay precision, three batches of the test strips were used to test the samples. The recovery and coefficient of variation (CV, %) were calculated from triplicate assays in all cases.



493

494 **Figure 1.** (A) Western blot analysis of mAb 2C3H5 with purified glycinin and
495 soybean protein isolate. Lane 1, pre-stained protein marker; Lane 2, purified glycinin;
496 and Lane 3, soybean protein isolate. (B) Protein profile of purified glycinin and
497 isolated soybean protein determined by SDS-PAGE. Lane 4, pre-stained protein
498 marker; Lane 5, purified glycinin; and Lane 6, isolated soybean protein. Subunits of
499 β -conglycinin and polypeptide chains of glycinin are shown on the right (Ax indicates
500 A1, A2 and A4).



501

502 **Figure 2.** (A) Sample extracts added with glycine standard solutions were tested

503 using the test strips, and the test lines were identified with the naked eye. (B) Relative

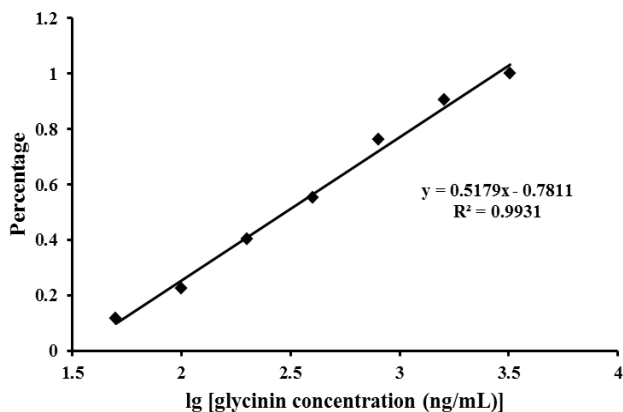
504 optical density (ROD) curves of the sample extracts. The extracts added with glycine

505 standard solutions at 0, 50, 100, 200, 400, 800, 1600, 3200, 6400, and 12800 ng/mL

506 were tested using the test strips. Test lines were scanned with a TSR3000 membrane

507 strip reader.

508



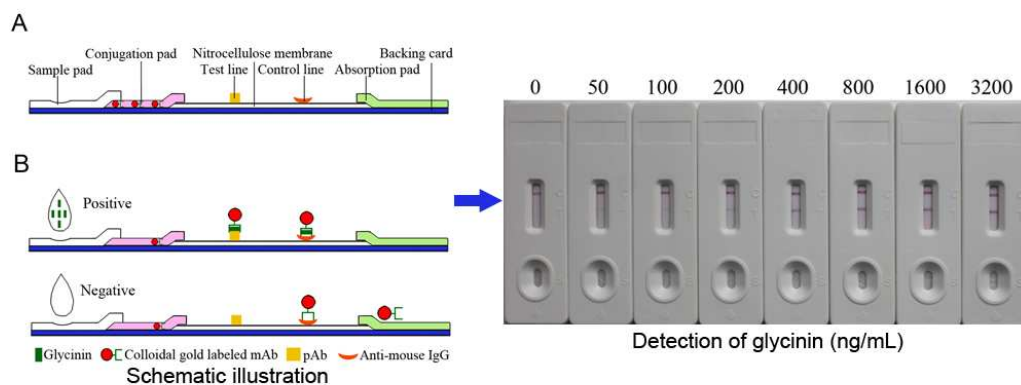
509
510 **Figure 3.** Standard curve for glycinin using test strip detection. The *X*-axis represents
511 the logarithmic concentration. The *Y*-axis represents the percentage of
512 $G/D \times \text{Area-ROD}$ of the standards divided by the $G/D \times \text{Area-ROD}$ at saturation (3200
513 ng/mL). The linear regression correlation coefficient (R^2) is 0.9931.

514



515
516 **Figure 4.** Soybean β-conglycinin, agglutinin, trypsin inhibitor, PNA, OVA, and casein,
517 each at a concentration of 20 μg/mL, were tested by test strip detection.

518

519 **Graphic for Table of Contents**

520