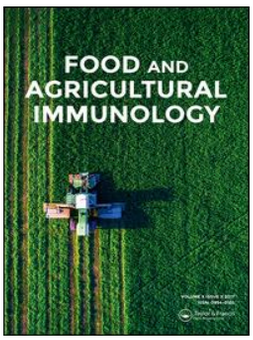

Food and Agricultural Immunology

ISSN: 0954-0105 (Print) 1465-3443 (Online) Journal homepage: https://www.tandfonline.com/loi/cfai20

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To cite this article: Yao Wang, Yanhong Li, Jiabei WU, Yafeng Pei, Xiujin Chen, Yaning Sun, Mei Hu, Yunrui Xing, Jinbo Cao, Zhaozhou Li, Peng Fei, Ruiguang Deng, Shaobin Gu & Xiaofei Hu (2019) Development of an immunochromatographic strip test for the rapid detection of soybean Bowman-Birk inhibitor, Food and Agricultural Immunology, 30:1, 1202-1211, DOI: 10.1080/09540105.2019.1680613

To link to this article: https://doi.org/10.1080/09540105.2019.1680613

Published online: 22 Oct 2019.

FOOD AND AGRICULTURAL IMMUNOLOGY
2019, VOL. 30, NO. 1, 1202–1211
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Development of an immunochromatographic strip test for the rapid detection of soybean Bowman-Birk inhibitor

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ABSTRACT

Bowman-Birk inhibitor (BBI) is the main type of soybean trypsin inhibitor, which is a kind of antinutritional factor. It can cause indigestion and hinder growth and development of human and animal. Herein, a rapid immunochromatographic strip was developed in a sandwich format using the anti-BBI monoclonal antibody (mAb) in order to specifically detect BBI. The mAbs showed high affinity towards BBI and no cross-reactivity with other soybean allergenic proteins and antinutritional factors. The assay could be performed within 10 min. The limit of detection of the test strip was 0.5 μg/mL for visual and 0.23 μg/mL for strip reader. The assay showed high specificity for BBI and recovery efficacy of BBI in spiked milk samples ranged between 83.24% and 89.86% with the coefficient of variation of less than 7.98%. Therefore, the test strip could be used as a rapid and reliable detection method for BBI qualitative or quantitative assessment.

ARTICLE HISTORY
Received 3 September 2019
Accepted 7 October 2019

KEYWORDS
Bowman-Birk inhibitor; monoclonal antibody; immunochromatographic strip

Introduction

Soybean is a rich source of protein and an important raw material used for processing food and feed. However, it also contains a variety of allergenic proteins and antinutritional factors (Wang, Qin, Sun, & Zhao, 2014). One of them is Soybean trypsin inhibitor (STI), which can not only induce a hypersensitive reaction, but can also inhibit the proteolytic activity of trypsin and chymotrypsin, leading to indigestion and hindered growth and development of human and animal (Liu et al., 2019). Two main types of STI are Kunitz trypsin inhibitor (KTI) and Bowman-Birk inhibitor (BBI). KTI has a molecular weight of approximately 20–25 kDa and can be combined with trypsin in equal

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proportions. KTI is insoluble in ethanol, sensitive to heat and acid, and can be easily inactivated (Chen, Xu, Zhang, Kong, & Hua, 2014). BBI has a molecular weight of approximately 6–10 kDa and contains two independent active sites. Thus, it is also known as a double-headed inhibitor, which can be combined with trypsin and chymotrypsin in equal proportion or combined with both at the same time. BBI is insoluble in acetone, resistant to heat, acid and enzyme hydrolysis, and relatively stable as compared with KTI (He, Li, Kong, Hua, & Chen, 2017).

Therefore, it is important to develop a sensitive and efficient detection method for the STI. Previously, immunoassays, due to their high sensitivity and specificity, have been used to detect STI. Monoclonal antibodies (mAbs) have been developed against STI and used in enzyme-linked immunosorbent assays (ELISA) (Brandon, Bates, & Friedman, 2004, 1989, 1988). These methods have enabled a rapid qualitative and quantitative detection of STI in processed soy products such as infant formulas; however, this method requires more than 2 h to complete including multiple incubation and washing steps.

In the past decade, the immunochromatographic strip has become increasingly important in the qualitative or semi-quantitative detection of various analytes (Sun et al., 2018; Wang et al., 2017; Yang et al., 2018). This is attributed to its simplicity, rapidity, sensitivity, specificity, and cost-effectiveness (Chen et al., 2017b; Peng et al., 2017). However, the immunochromatographic strip is only used for the detection of KTI (Xu et al., 2016). There are no methods available for the detection of BBI, which is relatively stable than KTI. Because BBI has higher stability, it can be used as a marker for the detection of soybean components. Therefore, in this study, we generated mAb against BBI and developed a novel sandwich-format strip for the detection of BBI. This was used as a tool for monitoring food and feed products.

Materials and methods

Reagents and materials

BBI, KTI, Glycinin, β-conglycinin, Soybean agglutinin (SBA), Freund's complete/incomplete adjuvant (FCA/FIA), TMB, and mouse monoclonal antibody isotyping reagents were purchased from Sigma-Aldrich (St. Louis, MO, US); goat anti-rabbit IgG-HRP, PEG1500, FBS, DMEM, RPMI-1640, HAT, and HT medium were purchased from Solarbio (Beijing, China); nitrocellulose (NC) membranes, glass fibre, and absorbent pads were purchased from Millipore (Bedford, MA, US). Other reagents and solvents were of analytical grade or higher. Rabbit anti-mouse IgG antibody and Ultrapure water that were used in the experiments were produced in our laboratory.

In this study, 8-week-old female SPF BALB/c mice, obtained from the Henan Laboratory Animal Center (Zhengzhou, China) were raised at the Henan Key Laboratory of Animal Immunology (Zhengzhou, China) following the principles of the Animal Care and Use Committee of the Henan Academy of Agricultural Sciences.

Preparation of mAb

Four mice were immunized subcutaneously with 50 μg BBI. The first injection of immunogen was an emulsion of PBS and FCA; three booster injections were administered at 3-

week intervals using the same dose of the immunogen emulsified in FIA. The titre of the antisera was measured through indirect ELISA, and the anti-BBI activity, represented by 50% inhibitory concentration (IC_{50}), was measured by indirect competitive ELISA following a previously reported method (Wang et al., 2015). The mouse with the highest titre and anti-BBI activity received a fifth injection intraperitoneally, and then its spleen was used for cell fusion. Hybridomas secreting anti-BBI mAbs were generated by standard methods (Köhler & Milstein, 1975). Selected clones were subcloned using the method of limiting dilution. Ascitic fluid was harvested from the paraffin-primed BALB/c mice and mAb was purified from the antiserum by ammonium sulfate precipitation.

The isotype of the mAb was determined using mouse monoclonal antibody isotyping reagents. The affinity of the mAb (K_a) was measured following a previously described procedure (Beatty, Beatty, & Vlahos, 1987). The specificity of mAb was assayed using cross-reactivity (CR) and Western blot (Wang et al., 2015). IC_{50} values of mAb for competitors (other common soybean allergenic protein and antinutritional factor, Glycinin, β-conglycinin, SBA, KTI, etc.) were determined through indirect competitive ELISA. The cross-reactivities (CR) of the mAb were calculated using the following formula (Chen et al., 2017a, 2019): CR = (IC_{50} for BBI)/(IC_{50} for competitors).

Fabrication of the immunochromatographic strip

Colloidal gold was produced by the reduction of gold chloride with 1% sodium citrate (w/v) and colloidal gold solution was adjusted to pH 9.0 with 0.2 mol/L sodium carbonate, as described previously (Zhang et al., 2006). When preparing the colloidal gold-labelled capture mAb, the optimum ratio (1 μL/mL) of mAb solution to colloidal gold solution was determined according to a previous report (Song et al., 2011). Next, mAb was added to the colloidal gold solution in 0.02 mol/L sodium borate (pH 9.0) containing 10% BSA, with constant stirring for 30 min. The mixture was incubated at room temperature (25°C) for 10 min in order to stabilize the conjugate. The labelled mAb was washed with 0.02 mol/L sodium borate (pH 9.0) containing 10% BSA by repeated centrifugation at 12,000 rpm and 4°C for 30 min. Then, the labelled mAb was resuspended in 0.02 mol/L sodium borate (pH 9.0) containing 1% BSA, 3% sucrose, and 0.03% sodium azide.

The development of the immunochromatographic strip was based on the principle of sandwich immunoassay, which consisted of sample pad, conjugate pad, NC membrane, and an absorbent pad. Colloidal gold-labelled capture mAb, detection mAb, and rabbit anti-mouse IgG antibody were dispensed onto the conjugate pad, test line (T line) and control line (C line), respectively. The immunochromatographic strip was fabricated following our previously described procedure (Sun et al., 2014).

Test procedure and principle

One strip, containing 100 μL of standard or sample solution, was dropped onto the sample pad. It took approximately 10 min to complete a test. If the sample was positive, the solution reacted with the colloidal gold-labelled capture mAb and then bound to the detection mAb in the T line and formed a red-coloured line. Higher amounts of BBI in the sample resulted in a thicker red-coloured line. On the contrary, if the sample was negative,

there was no red colour in the T line. As a procedural control, a red-coloured line always appeared in the C line regardless of the presence of BBI in the sample.

Evaluation of the immunochromatographic strip

The sensitivity of the strip was determined by testing a series of diluted standard solutions containing various concentrations of BBI (0, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32 and 64 μg/mL). For semi-quantitative detection, the visual limit of detection (LOD) was defined as the lowest concentration that could produce colour at the T line based on direct observation. Using the TSR3000 Membrane Strip Reader (BioDot, USA), we scanned the G/D × area (graph density × area) – ROD of the T line that could provide a more quantitative detection. The strip reader gave different G/D × area-ROD of the T line for a series of diluted standard solutions. The standard curve was constructed by plotting the G/D × area-ROD percentage obtained from the standard solutions against the logarithmic concentrations. G/D × area-ROD percentage was G/D × area-ROD of the standard solutions divided by the G/D × area-ROD at saturation, which indicated the binding degree of BBI with the T line. The linearity of the analyte was assessed by the coefficient of determination (R^2) and the LOD was calculated from the regression equation. The specificity of the strip was determined by testing other soybean allergens and antinutritional factors (SBA, KTI, Glycinin, and β-conglycinin) at a high concentration (100 μg/mL).

To identify the accuracy of the test strip, we procured commercially skimmed milk powder (YILI INC.) without soybean ingredients included in label, to evaluate the extent of BBI recovery by the strip test. Skimmed milk powder was diluted to 5% (w/v) using normal saline and spiked with BBI (5, 10, and 20 μg/mL representing low, medium and high levels). Next, the levels were detected by the strips. The ROD of the T line was measured using the Strip Reader, and BBI values were calculated from the standard curve.

Results and discussion

Characterization of the mAbs

After cellular fusion and screening by ELISA, two sensitive hybridomas, namely 7G7-D11 and 3B7-B10, were intraperitoneally injected into the mice in order to produce ascites. Next, the mAbs were purified from the ascitic fluid by the ammonium sulfate precipitation. The titres of 7G7-D11 and 3B7-B10 were both above $1{:}4.096 \times 10^5$; the affinity constant (Ka) of 7G7-D11 and 3B7-B10 were 1.05×10^8 and 1.13×10^8 L/mol, respectively. The isotype for both 7G7-D11 and 3B7-B10 was identified as IgG1, which was consistent with the commercially available antibody for specific detection of BBI (Muzard, Fields, O'Mahony, & Lee, 2012).

The CR results presented in Table 1, indicated that CR of the mAbs were remarkably low with SBA, KTI, Glycinin, and β-conglycinin. Western blot results showed that both 7G7-D11 and 3B7-B10 had a single band, with a molecular weight of approximately 10 kDa, corresponding to BBI (Figure 1). These results indicated that the mAbs reacted with BBI specifically without cross-reactivity with other soybean allergenic proteins and antinutritional factors.

Table 1. Cross-reactivity (CR) of mAbs.

Competitors	7G7-D11		3B7-B10	
	IC_{50} (ng/mL)	CR (%)	IC_{50} (ng/mL)	CR (%)
BBI	186.81	100	83.07	100
Glycinin	$>1.0 \times 10^4$	<2	$>1.0 \times 10^4$	<1
β-conglycinin	$>1.0 \times 10^4$	<2	$>1.0 \times 10^4$	<1
SBA	$>1.0 \times 10^4$	<2	$>1.0 \times 10^4$	<1
KTI	$>1.0 \times 10^4$	<2	$>1.0 \times 10^4$	<1

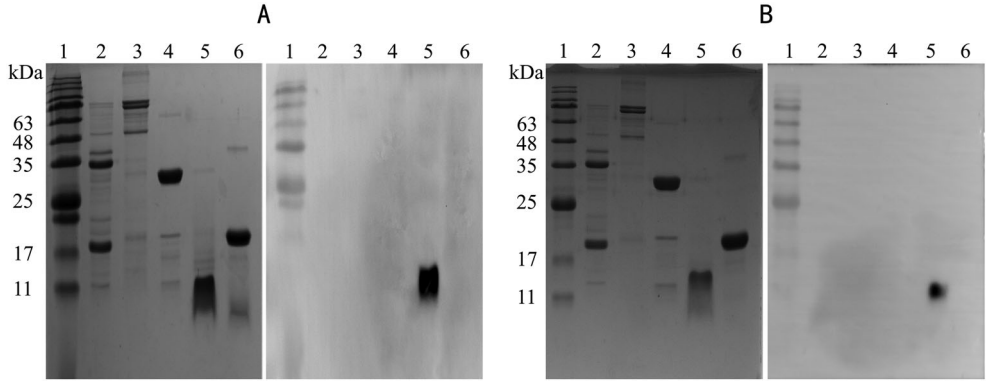

Figure 1. (A) Western blot analysis of mAb 7G7-D11, (B) Western blot analysis of mAb 3B7-B10. Both the left images are SDS-PAGE, right images are Western blot; Lane 1, pre-stained protein marker; line 2, glycinin; line 3, β-conglycinin; line 4 SBA; line 5, BBI; line 6, KTI.

Optimization of the colloidal gold-labelled capture mAb and detection mAb

In order to optimize the use of 7G7-D11 and 3B7-B10 as capture/detection mAb, both were used as colloidal gold-labelled capture mAb and dispensed onto the conjugate pad; and as detection mAb and dispensed onto the T line. Both were cross used to fabricate the strip. Next, the standard BBI solution (50 μg/mL) was used to test the strips and the process was repeated three times. As shown in Figure 2, while using 7G7-D11 as the capture mAb for colloidal gold labelling, both 7G7-D11 and 3B7-B10 could be used as detection mAb. Test results indicated that when the 3B7-B10 was used both as capture mAb and detection mAb, T line showed a stronger red-coloured band. Therefore, 3B7-B10 was used to fabricate the immunochromatographic strip.

Sensitivity of the test strip

The sensitivity of the strip was determined by testing a series of diluted BBI standard solutions. The results are shown in Figure 3. When standard solutions were applied to the strips, the colour of the T line appeared at the concentration of 0.25 μg/mL and the visual LOD was defined at 0.5 μg/mL to facilitate the T line colour identification during strip test. T line of the strips were scanned by TSR3000 Membrane Strip Reader and it was observed that the (G/D × area – ROD) values increased as the BBI concentration increased and became saturated after 32 μg/mL (Table 2 and Figure 3). A

Figure 2. Optimization of the colloidal gold-labelled capture mAb and detection mAb. On the absorbent pad, the number labelled in the upper row represents the detection mAb dispensed onto the T line, and the number labelled in the lower row represents the colloidal gold-labelled capture mAb dispensed onto the conjugate pad (No. 1 represents 7G7-D11, No. 2 represents 3B7-B10).

quantitative standard curve was constructed by plotting the logarithmic concentration of the standard BBI against the G/D × area – ROD percentage, which showed good linearity over the range of 0.25–32 μg/mL (Figure 4). Based on the regression equation, the LOD was quantitatively defined as the amount of BBI in the standard solution that caused a 10% increase in the G/D × area – ROD percentage produced by the 0 ng/mL BBI solution. Thus, the LOD of the strip was calculated to be 0.23 μg/mL. Although this strip is not as sensitive as the existing ELISA-based methods for detection of BBI (Brandon & Friedman, 2002; Wan, Lu, Anderson, Ware, & Kennedy, 2000), it is more rapid and simple to operate.

Specificity of the test strip

Standard solutions of SBA, KTI, Glycinin, and β-conglycinin, each at a concentration of 100 μg/mL, were tested using the strips (Figure 5). The T lines showed no colour, similar to the negative samples. These results indicated that the strip was highly specific for BBI and showed no cross-reactivity with other soybean allergenic proteins and antinutritional factors.

Recovery from the spiked samples

Milk samples spiked with BBI (5, 10, and 20 μg/mL) were used to test a single batch of strips ($n = 6$). The optical density of the T line was measured using the strip reader and

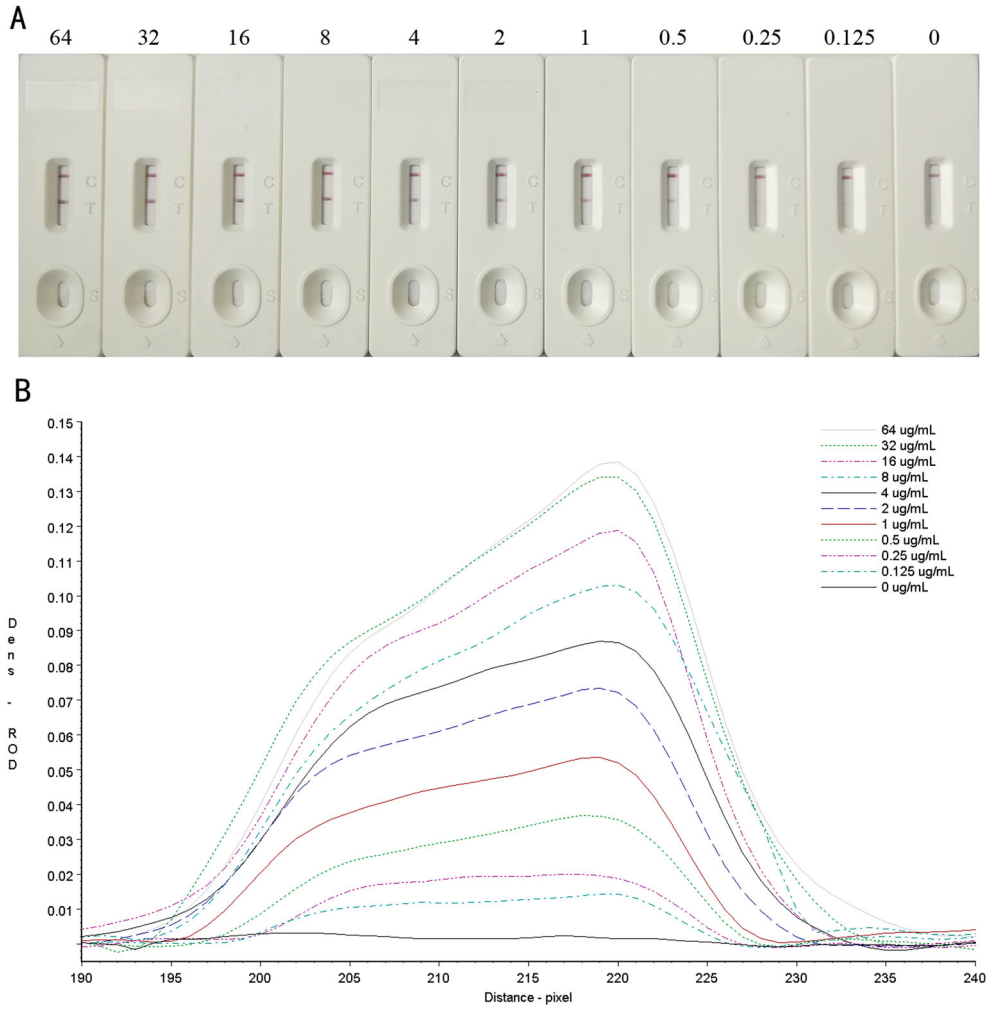

Figure 3. (A) Series of diluted BBI standard solutions were tested using the strips. (B) ROD curves of the standard solutions at 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125 and 0 µg/mL were tested using the strips.

Table 2. G/D × area-ROD of the T lines of the series of diluted BBI standard solutions.[a]

BBI concentration (µg/mL)	G/D × area-ROD (pixel)
0	1.7149
0.125	9.2308
0.25	13.1703
0.5	23.4145
1	37.0983
2	53.6510
4	65.6298
8	76.4705
16	88.5419
32	98.2509
64	98.3476

[a]The assays were carried out in triplicate using the test strips.

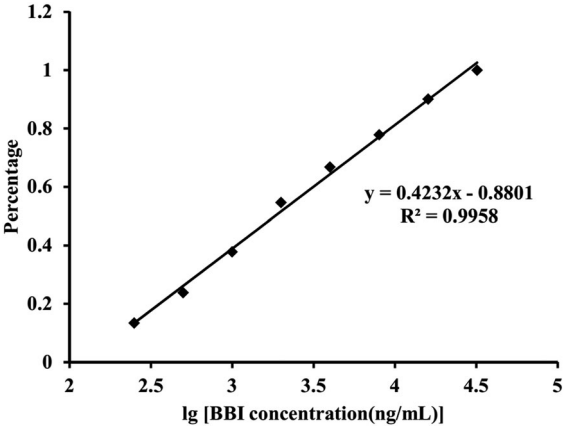

Figure 4. Standard curve for BBI using strip detection. The *X*-axis represents the logarithmic concentration. The *Y*-axis represents the percentage of G/D × area-ROD of the standards divided by the G/D × area-ROD at saturation.

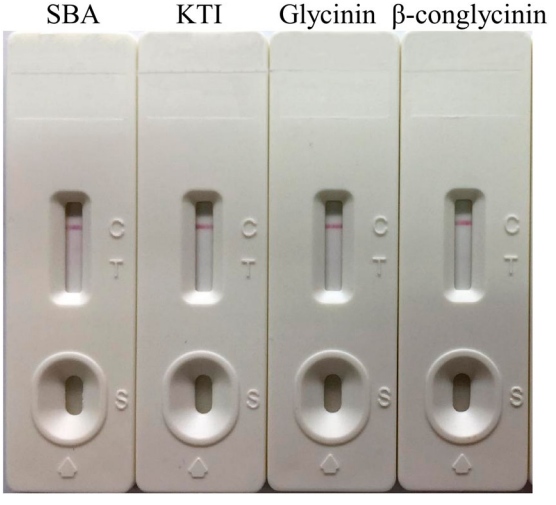

Figure 5. SBA, KTI, Glycinin and β-conglycinin solutions were tested by strips.

BBI concentrations were calculated from the standard curve. The recoveries ranged from 83.24% to 89.86% (Table 3), with the highest coefficient of variation (CV) as 7.98%, which met the analytical requirements.

Table 3. Recovery of the strips for BBI spiked milk samples.

Spiked BBI (μg/mL)	Mean ± SD (μg/mL)	Recovery (%)	CV (%)
5	4.16 ± 0.18	83.24	4.36
10	8.56 ± 0.55	85.65	6.45
20	17.97 ± 1.43	89.86	7.98

Conclusions

A novel immunochromatographic strip for the detection of BBI was established and evaluated. It was fabricated using the sandwich format by specific mAb for the rapid and sensitive detection of BBI in milk samples. The strips could be used for visual (qualitative) or strip reader (quantitative) assessment. The visual and strip reader LODs were 0.5 and 0.23 μg/mL, respectively. The recovery measurements of the strip test indicated that it was sufficient for the determination of BBI in powdered milk. Additionally, this strip could also be used to detect BBI in other processed foods and feeds after appropriate extraction of the samples. Moreover, this assay might be a valuable tool in identifying the effective approach for reducing the content of allergenic proteins and antinutritional factors.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the National Natural Science Foundation of China [grant number 31702218]; Natural Science Foundation of Henan Province [grant number 182300410038]; Scientific and Technological Research Project of Henan Province [grant numbers 182102110445, 182102310230]; Research Project of Henan Provincial Key Laboratory of Animal Immunology [grant number PKLAI20170603]; Key Scientific Research Project of Higher Education of Henan Province [grant number 18B550001].

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