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ARTICLE

Advantages of the Lab-on-a-Chip Method in the Determination of the Kunitz Trypsin Inhibitor in Soybean Varieties

Aleksandra M. Torbica,*[†] Dragan R. Živančev,[†] Zorica T. Nikolić,[‡] Vuk B. Đorđević,[‡] and Branislava G. Nikolovski[§]

[†]Institute for Food Technology, University of Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia, [‡]Institute of Field and Vegetable Crops, Maksima Gorkog 30, 21000 Novi Sad, Serbia, and [§]Faculty of Technology, University of Novi Sad, Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia

Qualitative and quantitative determination of the Kunitz trypsin inhibitor (KTI) in soybean experimental lines is very important in processes of selecting and breeding of new varieties. The total enzyme activity assay, sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), and Lab-on-a-Chip (LoaC) method were used for the determination of the presence and quantity of KTI in 15 soybean experimental lines and varieties. From the total trypsin inhibitor enzyme assay, inhibitor activities were registered in all samples, even in a Kunitz variety that was a negative control. The SDS−PAGE method did not detect the presence of the KTI protein band in seven soybean experimental lines and Kunitz variety, while the LoaC method showed the absence of KTI only in the Kunitz variety sample. Results confirmed the superiority of the LoaC method over other two methods in selectivity and sensitivity when KTI determination is concerned. Relationships between the KTI content obtained by the LoaC method and total trypsin inhibitor enzyme activity were established and statistically confirmed.

KEYWORDS: Lab-on-a-Chip (LoaC) method; total enzyme activity; soybean Kunitz trypsin inhibitor (KTI)

INTRODUCTION

Soybean seed protease inhibitors belong to two families, Kunitz and Bowman−Birk, and have a major impact on nutritional values. The Kunitz trypsin inhibitor (KTI), the major trypsin inhibitor in soybean, is a small, monomeric, and non-glycosylated protein, about 21.5 kDa. It has been characterized as an anti-nutritional protein, food allergen in human consumption of soy proteins (*1*,*2*). The soybean KTI belongs to the family of all anti-parallel, β-sheet proteins that are highly resistant to thermal and chemical denaturation (*3*). The inhibitory activity is largely inactivated by conventionally applied heat treatments of soy flour, but 10−20% of the residual activity remains (*4*). The variation of protein profiles of KTIs in seeds of wild soybean and cultivated soybean, as well as genetic variations of KTI1-, KTI2-, and KTI3-related genes, has been reported by Natarajan et al. (*5*).

According to Di Pietro and Liener (*6*), the standard methods of measuring protease inhibitors in foods by enzyme assays often give inaccurate results, with processed samples having low residual activity.

Traditionally, sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) is a technique employed by biochemists to detect and characterize proteins (*7*). However, this electrophoretic method is time-consuming and consists of a number of necessary manual steps, such as staining, destaining, imaging, and analyzing (*8*).

*To whom correspondence should be addressed. Telephone: +381-21-485-3779. Fax: +381-21-450-725. E-mail: aleksandra.torbica@fins.uns.ac.rs.

On the other hand, microfluidic or Lab-on-a-Chip (LoaC) devices and their application to sensitive chemical and biological analyses have been reported over the past decade. This system has the potential for a fast, reliable, and automatable analysis in the clinical diagnosis (*9*), analysis of the antioxidant/radioprotective properties of herbal plants (*10*), quantitative estimation of ascorbic acid and amino acids present in single cells (*11*), and separation and quantitation of proteins (*12*−*14*). Thus, this technique has been reported to be a high-throughput, automated alternative to traditional SDS−PAGE.

The LoaC method for protein analysis allows for the integration of electrophoretic separation, staining, destaining, and fluorescence detection into a single process and for it to be combined with data analysis.

The chip-based protein assay allows for purity analysis, sizing, and relative quantitation based on internal standards or absolute quantitation based on user-defined standards. The chip-based protein analysis is comparable in sensitivity, sizing accuracy, and reproducibility to SDS−PAGE stained with standard Coomassie (*15*). To achieve this, the LoaC method uses non-covalently bound fluorescent dyes that bind to the SDS−protein complexes on the chip (*16*). The resolution and linear dynamic range are improved. Absolute quantitation accuracy and reproducibility is improved in comparison to SDS−PAGE and is comparable to batch-based quantitation methods, such as Lowry and Bradford. The LoaC system has several additional advantages over conventional SDS−PAGE, including fast and efficient separation of various analytes, from small ions to large biomolecules, providing a

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Table 1. Total Trypsin Inhibitor Activity (TIU mg^{-1}) Obtained by the Tripsin Inhibitor Enzymatic Assay, Content (%) of KTI Obtained by SDS−PAGE, and the Concentration (ng/μL) and Content (%) of KTI Obtained by the LoaC Method in 15 Soybean Genotypes

soybean genotypes	total trypsin inhibitor activity (TIU mg^{-1})	content of KTI by SDS−PAGE (%)	concentration of KTI by LoaC method (ng/μL)	content of KTI by LoaC method (%)
line 1	5.10 ± 0.63	0.000	63.600 ± 12.243	0.850 ± 0.239
line 2	11.50 ± 0.90	4.180 ± 0.309	113.000 ± 0.159	1.500 ± 0.000
line 3	9.10 ± 0.89	4.440 ± 0.541	182.950 ± 1.510	2.200 ± 0.000
line 4	5.00 ± 0.63	0.000	60.550 ± 2.146	0.800 ± 0.000
line 5	9.20 ± 0.87	3.640 ± 0.481	103.400 ± 0.398	1.550 ± 0.080
line 6	5.90 ± 0.63	0.000	48.075 ± 19.984	0.700 ± 0.184
line 7	5.80 ± 0.98	0.000	53.100 ± 0.989	0.850 ± 0.092
line 8	3.10 ± 0.64	0.000	87.525 ± 13.769	1.525 ± 0.457
line 9	7.60 ± 0.87	2.480 ± 0.498	225.025 ± 41.479	3.150 ± 0.459
line 10	8.00 ± 0.88	3.260 ± 0.359	138.750 ± 29.307	2.475 ± 0.457
line 11	4.30 ± 0.62	0.000	71.200 ± 1.272	0.800 ± 0.000
line 12, Kunitz variety	4.10 ± 0.66	0.000	0.000	0.000
line 13, Vojvodjanka variety	6.90 ± 0.63	2.690 ± 0.496	138.100 ± 1.272	2.750 ± 0.080
line 14	3.40 ± 0.77	0.000	49.950 ± 0.716	1.100 ± 0.000
line 15	8.50 ± 0.62	3.510 ± 0.757	140.400 ± 0.636	2.800 ± 0.000

feasible method for conducting numerous experiments in parallel while consuming little reagent and achieving even better results (*14*, *15*, *17*). The LoaC method is a promising technique for protein analysis in the future. However, more challenges still exist in sample preparation, such as the extraction of a target fraction from solid samples or from fragile and complex biological samples (*14*).

Our aim was to apply the three methods to detect enzyme activity (trypsin inhibitor assay) and quantify the amount of soybean KTI (SDS−PAGE and LoaC methods) in soybean experimental lines for estimation of valuable materials for future breeding programs of soybean cultivars with low levels of KTI.

MATERIALS AND METHODS

Materials. A total of 15 soybean experimental lines and varieties from Serbia were selected from the Institute of Field and Vegetable Crops, Soybean Breeding Program. The Kunitz variety, lacking trypsin inhibitor, served as a negative control, while the Vojvodjanka variety served as a positive control (**Table 1**). The trypsin inhibitor from *Glycine max* (L.) Merrill was purchased from Fluka (Germany).

Trypsin Inhibitor Assay. The trypsin inhibitor activity was measured by the method of Liu and Markakis (*18*), with slight modification. The trypsin inhibitor activity assay is based on the hydrolysis of benzoyl-D,L-arginine-*p*-nitroaniline (BAPA) by trypsin. Ground samples were mixed with distilled water 1:100 (w/v) for 1 h. Suspension was diluted (1:1) with buffer (0.05 M Tris-HCl and 0.01 M $CaCl_2$ at pH 8.2) and filtrated through filter paper. The final dilution was prepared in a way that 1 mL of the sample extract inhibited 30−70% of enzyme activity of the used trypsin standard. The assay reaction was prepared following 2 mL of BAPA, 1 mL of diluted sample suspension, and 0.5 mL of trypsin solution (16 μg/mL crystal calf trypsin). The tubes with a mixture of sample, trypsin standard, and BAPA were incubated at 37 °C, and the reaction was terminated after 10 min by the addition of 1 mL of 30% acetic acid (w/v). The increase in absorbance at 410 nm was followed. Absorbance of all samples was read in four experiments in triplicate. Values of TI activity are expressed in conventional trypsin inhibitor units (TIU).

Protein Extraction. A total of 40 mg of seed powder was extracted in 1 mL of extraction buffer (30 mM Tris-HCl at pH 8.0 containing 0.01 M β-mercaptoethanol). The samples were left for 1 h at room temperature, with vortexing every 10 min. The precipitate of the samples was removed by centrifugation at 11000*g* for 20 min at room temperature. The obtained supernatant, which contained the total soluble soybean proteins, was used to prepare samples for both SDS−PAGE and LoaC methods.

SDS−PAGE. SDS−PAGE was carried out according to the procedure of Laemmli (*19*) in 1.5 mm thick gels with 12% (w/v) running gel and 5% (w/v) stacking gel in a vertical electrophoresis unit (Carl Roth, Germany).

A total of 50 μL of extract was mixed with 50 μL of SDS sample buffer [0.15 M Tris-HCl at pH 6.8, 3% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 7% (v/v) glycerol, and 0.03% Bromophenol Blue] and heated for 3 min in a boiling water bath. The solution was cooled to room temperature, and 12 μL of each sample was loaded into a well. SDS−PAGE was performed at 90 mA per gel for 4 h. After electrophoresis, the gels were stained for 2 h using 0.1% (w/v) Coomassie Brilliant Blue R-250. After staining, the gels were destained using a 10% (v/v) acetic acid solution, until a clear background was achieved. A PageRuler Prestained Protein Ladder (10−170 kDa, Fermentas, Lithuania) was used as protein molecular-weight (MW) markers.

Scanning Densitometry. Dried gels were scanned, and ImageQuant TL software (Amersham Biosciences) was used for volume integration in data analysis to determine the total absorbance of protein bands. The apparent absorbance of each protein was obtained by subtracting the background absorbance from the total absorbance of the protein bands within the same gel volume. The relative amount of trypsin inhibitor was expressed as a percentage of the total protein amount in the same gel lane. All samples were analyzed at four separate gels, in duplication per each gel.

LoaC Method. The chip-based separations were performed on the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA) in combination with the Protein 230 Plus LabChip kit and the dedicated Protein 230 software assay on 2100 expert software. All chips were prepared according to the protocol provided with the Protein 230 LabChip kit. The channels of the chip have to be filled with a mixture of a sieving matrix (non-cross-linked linear format) and a fluorescent dye for detection. The channels are filled by pipetting 12 μL of the gel dye mixture into one of the wells and applying pressure with a syringe for 60 s. The Protein 230 dye is a blue fluorescent dye that interacts with the protein SDS micelles. The gel−dye mixture is also added to the other system wells, where it serves as a buffer reservoir during the separation. The sample preparation is comparable to SDS−PAGE. Sample buffer (2 μL) including lithium dodecylsulfate, a reducing agent (if applicable), and two internal standards (lower marker at −4.5 kDa and upper marker at 240 kDa) are added to a 4 μL sample and 84 μL of deionized water.

The samples are heat-denatured at 95−100 °C for 3−5 min before loading them onto the chip. The chip is then placed into the bioanalyzer. Once the chip is placed into the instrument, the electrodes touch the liquids in the well, forming an electric circuit. These electric circuits make it possible to move samples from the sample well into the channels and to perform injections into the separation channel. Each sample is sequentially separated in the separation channel and detected by laser-induced fluorescence detection (670−700 nm) within 45 s. The complete analysis of 10 protein samples, including sizing and quantitation, takes 25 min (including the start-up phase of the instrument). After completion of the chip run, the software offers the alternative of displaying results as quantitative profiles (as for conventional liquid chromatography) and also as simulated gel-electrophoresis patterns. Fractioning is size-based, and the profiles show the smallest proteins emerging first in the profiles but at the bottom of the gel patterns (according to the convention for SDS−PAGE). All samples were analyzed in triplicate.

Statistical Analysis. Statistical analysis was performed using XLSTAT 2009 (Addinsoft) software for data analysis and statistical solutions for Microsoft Excel. Beside descriptive statistics, the analysis of variance (ANOVA), and the Fisher's F test, the analysis of covariance (ANCOVA) was performed as well. In comparison to the classical linear regression model ANOVA, ANCOVA mixes qualitative and quantitative explanatory variables. Analysis of the differences between the categories was performed by the following tests: Tukey's honestly significantly different (HSD) test, Fisher's least significant difference (LSD) test, Dunn–Sidak's corrected t test, Dunnet's corrected t test (for comparison to a single control) Newman–Keuls' (Student–Newman–Keuls) method, and Ryan–Einot–Gabriel–Welsch (REGWQ) multiple range test.

RESULTS AND DISCUSSION

Protein samples isolated from 15 soybean experimental lines and varieties were analyzed by conventional (SDS–PAGE) and novel (LoaC) electrophoretic methods to detect and quantify the amount of soybean KTI. A sample of the commercial trypsin inhibitor was also subjected to the electrophoretic assays, and the protein bands in gel images corresponding to KTI served as the MW marker of KTI. Prior to the electrophoretic assays, the total trypsin inhibitor enzymatic assay was applied to protein extracts of the investigated soybean samples and the results were expressed in TIU. The Kunitz variety, lacking trypsin inhibitor, served as a negative control, while the Vojvodjanka variety served as a positive control for all applied methods.

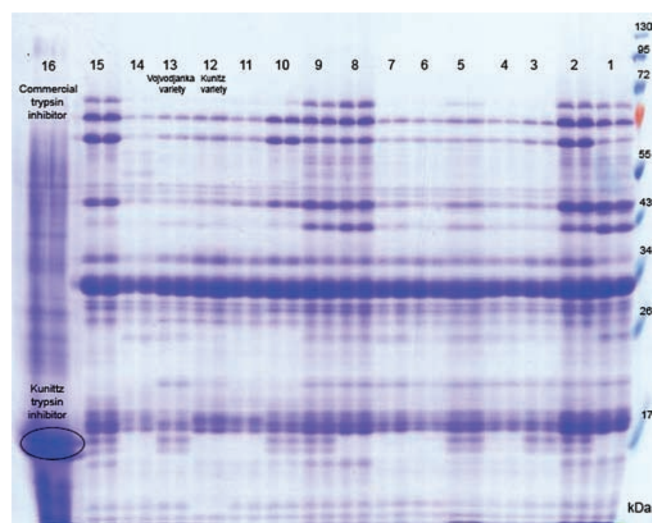

Figure 1. SDS–PAGE patterns of proteins from soybean samples (lanes 1–15), commercial trypsin inhibitor (lane 16), and MW markers (17, 26, 34, 43, 55, 72, 95, and 130 kDa). Lanes 12 and 13 represent Kunitz and Vojvodjanka varieties. Framed bands of commercial trypsin inhibitor sample (lane 16) have MWs of about 21.5 kDa, which is a typical MW of KTI.

In **Table 1**, the data of the total trypsin inhibitor activity (TIU mg^{-1}) obtained by the trypsin inhibitor enzymatic assay, the content (%) of KTI obtained by SDS–PAGE, and the concentration (ng/μL) and content (%) of KTI obtained by the LoaC method in examined soybean genotypes are presented. From the enzymatic assay, activities of the trypsin inhibitor were registered in all examined samples, even in the Kunitz variety (column 2 in **Table 1**).

The SDS–PAGE gel photograph of protein patterns from all soybean samples, commercial trypsin inhibitor, and MW markers is shown in **Figure 1**. The protein patterns obtained by the SDS–PAGE method possess lots of protein bands in the whole range of MWs. According to Quierce et al. and Burks et al. (*1*, *2*), we used bands of MW of about 21.5 kDa of commercial trypsin inhibitor (framed detail of lane 16 in **Figure 1**) as a MW marker of KTI. From that point of view, it is obvious that the SDS–PAGE gel shows the absence of the KTI band in seven soybean experimental lines (lanes 1, 4, 6, 7, 8, 11, and 14 in **Table 1**; lanes 1, 4, 6, 7, 8, 11, and 14 in **Figure 1**) and the Kunitz variety (lane 12 in **Table 1**; lane 12 in **Figure 1**). These results indicate that SDS–PAGE method possesses higher selectivity in comparison to enzymatic assay.

The LoaC gel image of all soybean samples, commercial trypsin inhibitor, and MW ladder is shown in **Figure 2**. Almost all protein bands of soybean samples from the LoaC gel image are in the same range of MWs as in the SDS–PAGE gel (**Figure 1**). However, the resolution of all protein bands, especially in the range of low MWs, is higher in comparison to SDS–PAGE, which is in agreement with the explanation by Kuschel et al. (*15*) about the different nature of SDS–PAGE (cross-linked) and LoaC (linear polymer) gels. This fact reperesents an advantage in the determination of KTI because its MW belongs to the range of low MWs. Also, the LoaC method detected the presence of the KTI peak in seven soybean experimental lines (framed detail in **Figure 3**), which lacked the KTI band in SDS–PAGE gel (lanes 1, 4, 6, 7, 8, 11, and 14 in **Figure 1**). This is a confirmation of a better sensitivity of the LoaC method in comparison to SDS–PAGE. Additionally, to emphasize a previous fact, in **Figure 4A** is shown overlaying LoaC profiles of the Kunitz variety (negative control), Vojvodjanka variety (positive control), and commercial trypsin

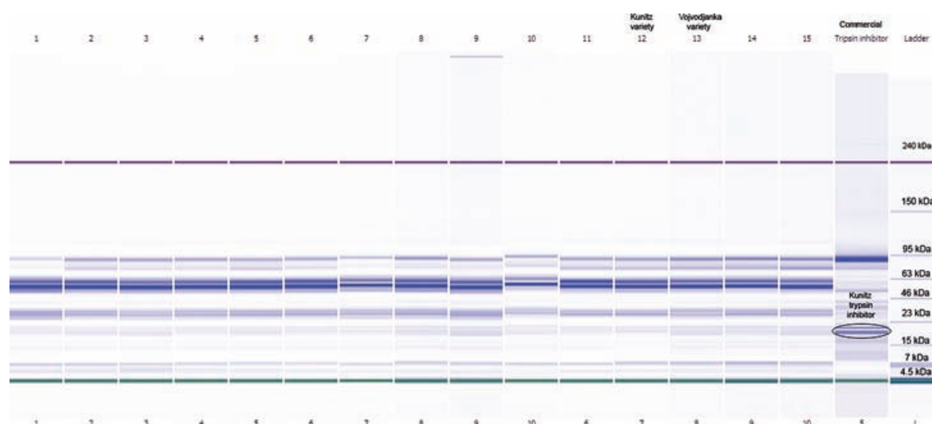

Figure 2. LoaC gel image of proteins from soybean samples (lanes 1–15), commercial trypsin inhibitor, and MW ladder (4.5, 7, 15, 23, 46, 63, 95, 150, and 240 kDa). Lanes 12 and 13 represent Kunitz and Vojvodjanka varieties. Framed bands of the commercial trypsin inhibitor sample have MWs of about 21.5 kDa, which is a typical MW of KTI.

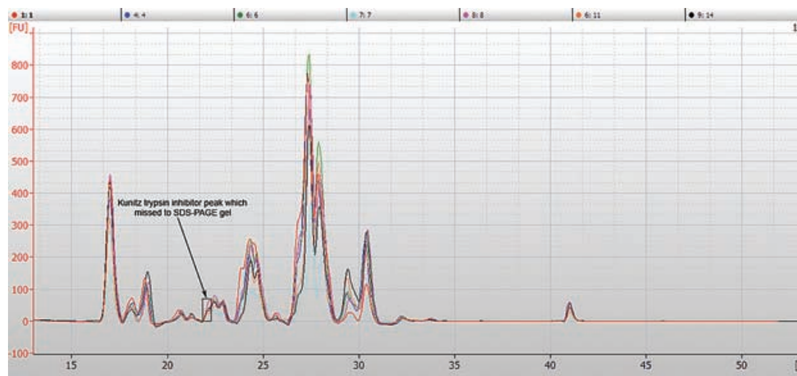

Figure 3. LoaC profiles of seven soybean experimental lines that showed the presence of the KTI peak (lanes 1, 4, 6, 7, 8, 11, and 14 in **Figure 1** showed the absence of the KTI band on the SDS−PAGE gel). Framed detail labels the KTI peak.

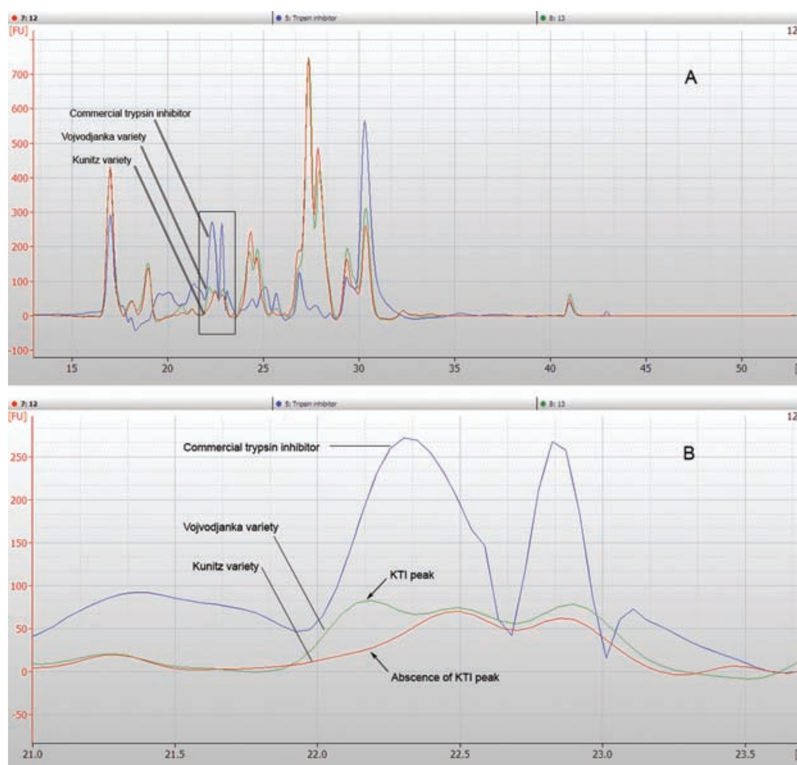

Figure 4. (A) Overlaying LoaC profiles of the Kunitz variety (negative control, red line), Vojvodjanka variety (positive control, green line), and commercial trypsin inhibitor sample (blue line). (B) Enlarged detail of the overlaying LoaC profiles of the Kunitz variety (negative control, red line), Vojvodjanka variety (positive control, green line), and commercial trypsin inhibitor sample (blue line) defines (framed profile area in panel A) the absence of one of the three peaks in the Kunitz variety sample in the range of KTI MW (21−24 kDa). These profiles show that the Vojvodjanka variety and commercial trypsin inhibitor sample possess a peak that is missing in the Kunitz variety.

inhibitor sample. The framed LoaC profile area of these three samples (**Figure 4A**) defines the absence of one of the three peaks in the Kunitz variety sample in the range of KTI MW (21−24 kDa) (*20*). Moreover, the enlarged detail in **Figure 4B** shows that the Vojvodjanka variety and commercial trypsin inhibitor sample possess a peak that is missing in the Kunitz variety. From software analysis, the obtained average MW of Kunitz TI bands was precisely 21.1 kDa. Furthermore, from the calculation by Byoanalizer 2100 expert software, the concentration of the KTI peak was higher in the commercial trypsin inhibitor sample (990 ng/μL) than in the Vojvodjanka variety (138.1 ng/μL). The above mentioned results confirmed the presence of KTI in the Vojvodjanka variety and its absence in the Kunitz variety. Therefore, undoubtedly, these two varieties can be used as positive and negative controls for testing experimental lines of soybean.

The existence of the peak that corresponds to the MW of KTI was determined by the LoaC method in all examined experimental soybean line samples. Nevertheless, the measured peak concentration divided samples into two groups, a group of soybean lines with a peak concentration of <100 ng/μL and a group whose peak concentration is >100 ng/μL (column 4 in **Table 1**). Analysis of the differences between the categories with a confidence interval of 95% was performed. All applied methods of comparison gave the same results of the standardized difference between the groups (5.4), which was compared to the critical value (2.2) and, consequently, confirmed the statistical difference between categories with the low peak concentration of the trypsin inhibitor (*KTI-L*) and the high peak concentration of the trypsin inhibitor (*KTI-H*) ($p < 0.0001$). Therefore, there was a need to introduce a qualitative independent variable that takes value *L* or *H*

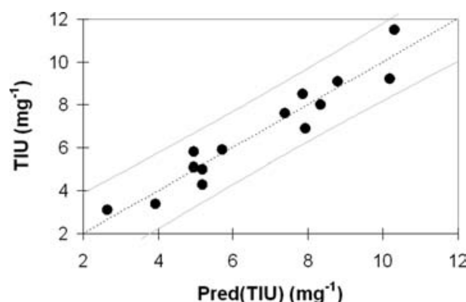

Figure 5. Predicted values versus the observed values of the total trypsin inhibitor activity obtained by the inhibitor assay (TIU mg^{-1}).

corresponding to an experimental soybean line sample being placed in the *KTI-L* or *KTI-H* group.

The ANCOVA can be used to find out how the content of KTI determined by the LoaC method (LoaC %) varies with the total trypsin inhibitor activity obtained by the inhibitor assay (TIU mg^{-1}) and the level of the peak concentration of KTI (a qualitative variable that takes value L or H) in soybean genotypes and to verify if a linear model is applicable after removing the variance for which quantitative predictors account. Results of ANCOVA show that 89% of the variability of the content of KTI obtained by the LoaC method (LoaC %) is explained by the total trypsin inhibitor activity obtained by the inhibitor assay (TIU mg^{-1}) and the level of the peak concentration of KTI > 100 or < 100 ng/μL. However, when ANCOVA is repeated in a way that the logarithm of the content of KTI obtained by the LoaC method is used as the dependent variable [ln(LoaC %)] and logarithm of the total trypsin inhibitor activity obtained by the inhibitor assay is used as the independent quantitative variable [ln(TIU mg^{-1})], better results are achieved. The model proposed as a result is

$$y = \underset{(\pm 0.337)}{1.550} - \underset{(\pm 0.219)}{1.083 \ln(\text{TIU})} + \underset{(\pm 0.165)}{1.596\text{KTI-H}}$$

where y represents the estimated value for the logarithm of the content of KTI obtained by the LoaC method [ln(LoaC %)] when the values ln(TIU) change inside the experimental range and a qualitative variable that takes value L or H and the values in parentheses are the standard errors of coefficients. The value of the coefficient of determination (R^2) is 0.924 (R^2_{adj} = 0.910), meaning that the lack of fit was not significant and that two selected variables can explain 92.4% of the variability of the logarithm of the content of KTI obtained by the LoaC method. Given the fact that the probability corresponding to the result of the ANOVA (Fisher's F test) is lower than 0.0001, it can be concluded with confidence that the two variables do give a significant amount of information, with approximately the same strong impact of the variables on the model.

An inverse model that can be employed to calculate the total trypsin inhibitor activity obtained by the inhibitor assay (TIU mg^{-1}) based on the content of KTI determined by the LoaC method (LoaC %) and the level of the peak concentration of the Kuniyz trypsin inhibitor > 100 or < 100 ng/μL determined by the LoaC method is

$$\text{pred(TIU)} = \underset{(\pm 0.291)}{4.314} - \underset{(\pm 0.779)}{3.932 \ln(\text{LoaC }\%)} + \underset{(\pm 0.811)}{7.589\text{KTI-H}}$$

The value of the coefficient of determination (R^2) is 0.920 (R^2_{adj} = 0.905), and the lack of fit was not significant ($p < 0.05$).

Figure 5 shows the predicted values versus the observed values of the total trypsin inhibitor activity obtained by the inhibitor assay (TIU mg^{-1}) as well as the confidence intervals.

Values predicted by the proposed model correspond well to the observed values of the total trypsin inhibitor activity obtained by the inhibitor assay (TIU mg^{-1}).

The same principle of statistical analysis was not introduced to the results of the SDS−PAGE method because of its low sensitivity and the fact that 7 of 15 experimental lines showed the absence of the KTI band.

The results presented in this study showed that the enzymatic assay possesses lower selectivity in comparison to SDS−PAGE and LoaC methods. The protein separation was better with the chip device than with the classic electrophoresis, which resulted in better resolution of the LoaC method. Also, the LoaC method had better sensitivity in comparison to the SDS−PAGE method. When a soybean line sample is categorized, in either the group with low KTI content (peak concentration < 100 ng/μL) or the group with high KTI content (peak concentration > 100 ng/μL), there is a linear relationship between the logarithm of the content of KTI obtained by the LoaC method and the logarithm of the total trypsin inhibitor activity obtained by the enzymatic assay (ANCOVA, R^2 = 0.924). All of the results mentioned above indicate that the LoaC method could be successfully applied for precise identification and quantitation of KTI in soybean cultivars. Additionally, this technique because of its fast and reliable analysis is especially useful for future breeding programs of soybean cultivars with a low level of tripsin inhibitor.

ABBREVIATIONS USED

ANCOVA, analysis of covariance; ANOVA, analysis of variance; BAPA, benzoyl-D,L-arginine-*p*-nitroaniline; HSD, honestly significantly different; KTI, Kunitz trypsin inhibitor; LoaC, Lab-on-a-Chip; LSD, least significant difference; MW, molecular weight; REGWQ, Ryan−Einot−Gabriel−Welsch; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TIU, conventional trypsin inhibitor units.

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LITERATURE CITED

(1) Quirce, S.; Nieto, F. M.; Polo, F.; Sastre, J. Soybean trypsin inhibitor is an occupational inhalant allergen. *J. Allergy Clin. Immunol.* **2002**, *109* (1), 178.
(2) Burks, A. W.; Cockrell, G.; Connaughton, C.; Guin, J.; Allen, W.; Helm, R. M. Identification of peanut agglutinin and soybean trypsin inhibitor as minor legume allergens. *Int. Arch. Allergy Immunol.* **1994**, *105*, 143–9.
(3) Roychaudhuri, R.; Sarath, G.; Zeece, M.; Markwell, J. Stability of the allergenic soybean Kunitz trypsin inhibitor. *Biochim. Biophys. Acta* **2004**, *1699*, 207–212.
(4) Friedman, M.; Brandon, D. L. Nutritional and health benefits of soy proteins. *J. Agric. Food Chem.* **2001**, *49*, 1069–1086.
(5) Natarajan, S.; Xu, C.; Bae, H.; Bailey, B. A. Proteomic and genomic characterization of Kunitz trypsin inhibitors in wild and cultivated soybean genotypes. *J. Plant Physiol.* **2007**, *64*, 756–763.
(6) Di Pietro, C. M.; Liener, I. E. Heat inactivation of the Kunitz and Bowman−Birk soybean protease inhibitors. *J. Agric. Food Chem.* **1989**, *37*, 39–44.
(7) Ozols, J. Amino acid analysis. *Methods Enzymol.* **1990**, *182*, 587–601.
(8) Hsieh, J. F.; Chen, S. T. Comparative studies on the analysis of glycoproteins and lipopolysaccharides by the gel-based microchip and SDS−PAGE. *Biomicrofluidics* **2007**, *1* (1), No. 014102.
(9) Wang, H.; Wang, D.; Wang, J.; Wang, H.; Gu, J.; Hana, C.; Jin, Q.; Xu, B.; He, C.; Li, C.; Wang, Y.; Zhao, J. Application of poly(dimethylsiloxane)/glass microchip for fast electrophoretic

separation of serum small, dense low-density lipoprotein. *J. Chromatogr., A* **2009**, *1216*, 6343–6347.
(10) Pravin, N. K.; Kwon, K. T.; Song, M. J. Development of a photosensitive, high-throughput chip-based superoxide dismutase (SOD) assay to explore the radioprotective activity of herbal plants. *Biosens. Bioelectron.* **2009**, *24*, 3587–3593.
(11) Zhao, S.; Huang, Y.; Yi-Ming, L. Microchip electrophoresis with chemiluminescence detection for assaying ascorbic acid and amino acids in single cells. *J. Chromatogr., A* **2009**, *1216*, 6746–6751.
(12) Goetz, H.; Kuschel, M.; Wulff, T.; Sauber, C.; Miller., C.; Fisher, S.; Woodward, C. Comparison of selected analytical techniques for protein sizing, quantitation and molecular weight determination. *J. Biochem. Biophys. Methods* **2004**, *60*, 281–293.
(13) Hey, J. S. Advances in microscale protein fractionation and analysis. *Am. Biotechnol. Lab.* **2007**, *25*, 12–16.
(14) Wu, D.; Qin, J.; Lin, B. Electrophoretic separations on microfluidic chips. *J. Chromatogr., A* **2008**, *1184*, 542–559.
(15) Kuschel, M.; Neumann, T.; Barthmaier, P.; Kratzmeiera, M. Use of Lab-on-a-Chip technology for protein sizing and quantitation. *J. Biomol. Technol.* **2002**, *13*, 172–178.
(16) Bousse, B.; Mouradian, S.; Minalla, A.; Yee, H.; Williams, K.; Dubrow, R. Protein sizing on a microchip. *Anal. Chem.* **2001**, *73*, 1207–1212.
(17) Hawtin, P.; Hardern, I.; Wittig, R.; Mollenhauer, J.; Poustka, A. M.; Salowsky, R.; Wulff, T.; Rizzo, C.; Wilson, B. Utility of Lab-on-a-Chip technology for high-throughput nucleic acid and protein analysis. *Electrophoresis* **2005**, *26*, 3674–3681.
(18) Liu, K.; Markakis, P. An improvement colorimetric method for determining antitryptic activity in soybean products. *Cereal Chem.* **1989**, *66* (5), 415–422.
(19) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227* (5259), 680–685.
(20) Struthers, B. J.; MacDonald, J. R. Comparative inhibition of trypsins from several species by soybean trypsin inhibitors. *J. Nutr.* **1983**, *113*, 800–804.

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