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Analytical Methods

Continuous method to determine the trypsin inhibitor activity in soybean flour

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ABSTRACT

The determination of trypsin inhibitor (TI) activity is of importance to evaluate the nutritional value of soybean flours. An analytical method, which involves a continuous spectrophotometric rate determination for trypsin activity against the substrate N-benzoyl-DL-arginine p-nitroanilide, is proposed as an alternative to the standard discontinuous assay. Stopping the reaction with acetic acid and a centrifugation/filtration step to decrease turbidity are not required, thus reducing costs and sample preparation time.

The TI activity of different flour samples, determined by both assays, demonstrated to be statistically comparable, irrespective of the TI concentration level. The coefficients of variation of the novel method did not exceed 8% at any concentration level.

The curves of progress reaction showed a non-linear behavior in samples without TI. A reduction of incubation time from 10 min to 2 min increased the method sensitivity and extended its linear range.

A more economical, faster and simpler assay was developed.

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1. Introduction

At present, soybean is the world's most important oilseed, used not only for the extraction of oil but for the recovery of protein as well (Day, 2013). Soybean industry spans continents, feeds millions of livestock, and takes part as ingredient in most of our diet. The high nutritional value of soybean protein is determined by its composition, which includes all the essential amino acids required for growth and by its excellent bioactive properties such as antioxidant, antihypertensive, antithrombotic, hypocholesterolemic (Alibhai, Mondor, Moresoli, Ippersiel, & Lamarche, 2006; Coscueta et al., 2016; Isanga & Zhang, 2008; Wang, Mejia, & Gonzalez, 2005). However, like other beans and grains, soybean contains several components (lectins, protease inhibitors, etc.) related to protection and immune mechanisms in the plant, which prevent the utilization and digestibility of soybean protein (Bajpai, Sharma, & Gupta, 2005; Becker-Ritt, Mulinari, Vasconcelos, & Carlini, 2004; Norton, 1991). Among these anti-nutritional factors, the trypsin inhibitors (TI) are the major components reported to cause retardation of growth and digestive and metabolic diseases (Gatel, 1994; Norton, 1991; Westfall & Hauge, 1948). These factors are heat labile, therefore, they are able to be removed totally or partially by different treatments during soybean industrialization (Akande & Fabiyi, 2010; Babar, Chavan, & Kadam, 1988; Elsheikh, Fadul, & El Tinay, 2000; van den Hout, Pouw, Gruppen, & van't Riet, 1998). The determination of TI activity is of importance to evaluate the nutritional value of soybean flour after processing stages in order to provide high-quality products. At present, the standard method is based on the ability of extracts of soybean flours to inhibit the activity of trypsin towards the chromogenic substrate N-benzoyl-DL-arginine p-nitroanilide (Kakade, Rackis, Mc Ghee, & Puski, 1974). The amount of the product p-nitroaniline, formed during a 10 min incubation step, is determined through absorbance measurements in the presence and absence of soybean extract, thus giving differences related to the TI activity. Results are expressed in trypsin inhibitor units (TIU) per gram of initial soybean sample, each TIU being defined as the amount of inhibitor that causes a change of 0.01 in absorbance units at 410 nm per 10 mL of reaction mixture under the experimental conditions (AOCS, 2009). It is a discontinuous or stopped assay in which the enzyme (trypsin) is inactivated after

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the incubation time (10 min) by lowering the pH with the addition of acetic acid. The overall assay is complicated and does not fulfill the requirements for quality control in industrial processing of soybean derivatives. It presents several shortcomings (Liu & Markakis, 1989; Stauffer, 1990), as addressed bellow. The stopping reactant (acetic acid) causes the denaturation not only of the trypsin but also of the extract proteins, thus producing the formation of turbidity which must be reduced by a later filtration/centrifugation step. A blank, in which the substrate is added after the acid, is required to be prepared in order to subtract the remained turbidity. Each determination involves the preparation of several trial dilutions, attempting to arrive at a concentration in which the sample inhibition is between 40% and 60% of total trypsin activity in order to minimize the relative standard deviation. Time delay steps between additions are required to reach identical reaction conditions (incubation/stopping times), thus limiting the number of samples able to be analyzed simultaneously. Finally, this assay presents a disadvantage proper of any discontinuous one, the shape of the progress curve is not revealed and any irregularity in curvature is not able to be detected (Meyers, 1995).

In this context, the goal of this work was to overcome the above mentioned disadvantages by developing an improved continuous assay as a potential replacement of the current one. An adequate treatment of the data was proposed to inform the results in TIU units comparable with those obtained from the standard stopped method. Furthermore, the analysis of the progress of reaction with time was assessed to determine the optimal conditions that assure a linear relationship between the measurements and the TI activity in samples.

2. Materials and methods

2.1. Materials

Crystallized, salt free trypsin (bovine) (TRP) and α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) were purchase from Sigma Chem. Co. and used without further purification. All other reagents were of analytical quality.

Different soybean flours were analyzed. Defatted soybean flours, both deactivated and non-deactivated samples, were obtained from the food processing company Molinos Río de la Plata SA (San Lorenzo, Argentina). An additional commercial sample of soybean flour was purchased from a local market.

2.2. Procedures

2.2.1. Deactivation

Several soybean flours were subjected to mild and moderate deactivation treatments in our laboratory in order to obtain samples with different TI activity and extend the analyzed range. Heating protocols were carried out by incubating the samples in an oven at two temperatures (80, 100 °C) for different periods of time (1, 1.5 and 2.5 h). Each sample and its treatment are described below:

- Sample 1: Defatted soybean flour without any thermal deactivation treatment, supplied by Molinos Río de la Plata SA.
- Sample 2: Defatted soybean flour supplied by Molinos Río de la Plata SA, subjected to oven dry heat at 80 °C for 1 h.
- Sample 3: Defatted soybean flour supplied by Molinos Río de la Plata SA, subjected to oven dry heat at 100 °C for 1.5 h.
- Sample 4: Defatted soybean flour thermally deactivated by Molinos Río de la Plata SA.
- Sample 5: Commercial defatted soybean flour.
- Sample 6: Commercial defatted soybean flour subjected to oven dry heat at 100 °C for 2.5 h.

2.2.2. Preparation of TI extracts

The extraction of TI was performed by mixing 1.00 g of soybean flour (treated/non-treated) with 50.0 mL of 0.01 M NaOH and agitating the resultant suspension for 3 h at room temperature according to the standard method proposed by Kakade et al. (1974) and later modified (AOCS, 2009). A final centrifugation step for 10 min at 3500 rpm allowed separating the supernatant (soybean flour extract) for the TI assay.

2.2.3. Discontinuous TI assay

The reaction between TRP and BAPNA in absence and presence of TI (soybean flour extracts) was performed for 10 min at 37 °C and stopped by acetic acid. The reaction mixture was centrifuged for 15 min at 10,000 rpm and filtered through a Watman No. 2 paper in order to obtain a clear supernatant. The extension of the reaction was followed by the absorbance of the released reaction product, the p-nitroaniline, at 410 nm. The preparation of TRP and BAPNA solutions, all the protocol steps and the final calculations were carried out by following exactly the AOCS method (AOCS, 2009). Results were expressed in trypsin inhibitor units (TIU) per gram (g) of soybean flour, TIU/g.

Each TIU is arbitrarily defined as the change of 0.01 absorbance units at 410 nm per 10 mL of reaction mixture after 10 min of reaction in Tris buffer pH 8.20, 0.050 M at 37 °C.

2.2.4. Continuous TI assay

The modified proposed method involves a continuous spectrophotometric rate determination for trypsin activity against the substrate BAPNA, the reaction taking place directly in the spectrophotometer cuvette with a reduction of volume from 10 to 2.5 mL. Stopping the reaction with acetic acid and clarification step to reduce turbidity were not required. Nevertheless the final concentrations (in cuvette) of all the reactants -enzyme, substrate and buffer- were the same of the standard method, the concentrations and volumes of working solutions were modified to improve the procedure and make it simpler. All the details of the modified protocol are given below:

2.2.4.1. Reactants. Stock and working solutions of TRP and BAPNA were prepared according to the following directions.

The *TRP stock solution* was prepared by dissolving 10 mg of TRP in 1 mL of 0.001 M HCl acid. It was stored at −18 °C until use and mixed gently when defrosting. The *TRP working solution* was prepared fresh daily by a 1:100 dilution of *TRP stock solution*, defrosted and gently mixed with Tris buffer 0.050 M pH 8.20. This solution was maintained in an ice-water bath during the assay.

The *BAPNA stock solution* was prepared by dissolving 100 mg of solid BAPNA in 2.3 mL dimethyl sulfoxide. It was maintained at −18 °C until use. The *BAPNA working solution* was prepared fresh daily by a dilution 1:100 of the *BAPNA stock solution*, previously defrosted and gently mixed, with Tris buffer 0.050 M pH 8.20.

2.2.4.2. Rate measurements. Each TI determination required of two conditions to be measured: Control (trypsin activity without inhibitor) and Sample (trypsin activity in the presence of inhibitor). The volumes (mL) of reagents/samples and their sequence of addition are indicated in Table 1.

Immediately after mixing, the Absorbance at 410 nm was monitored for 10 min recording measurement readings at time intervals of 10 s or less. The reaction rate (Abs units/min) was obtained from the slope (m) of Absorbance vs. time plot at both conditions ($m_{Control}$, m_{Sample}). The assays were performed at constant temperature of 37 °C.

Notice that the standard method states that the rate at Sample condition must be 0.4–0.6 times the value of the Control one to

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