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Ultrasound-based protein determination in maize seeds

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ABSTRACT

The need for a simple and accurate method for protein estimation in alcoholic extracts led to the reexamination of the optimum conditions of a colorimetric assay based on the biuret reaction. Sonication time and the other experimental parameters were optimized after kinetics study on the extraction of either zein or total proteins. Zein extraction and purity were investigated by ^{1}H and ^{13}C NMR spectroscopy, SDS–PAGE electrophoresis, and UV–visible spectrophotometry (UV–vis). A zein assay was proposed, which involves the reaction of copper ions in copper phosphate powder with zein extracted in ethanolic solutions under strong alkaline environment. Furthermore, we extended this procedure to determine total proteins in maize samples simultaneously with their ultrasonic-assisted (US) extraction with an alkaline–alcoholic solution. Proteins in both types of extracts were well characterized by UV–vis spectroscopy. However, the 545 nm absorbance of the violet-colored supernatants which is proportional to the protein content was found to be the key parameter of the improved biuret-based protein assay. Comparison of values obtained by this procedure and by Micro-Kjeldahl method was in excellent agreement. A scaled-down procedure agreed well with the standard procedure. Enhanced accuracy and repeatability was found in protein determination in maize using the modified biuret method. The optimization of reagent concentrations and incubation times were studied as well.

1. Introduction

Ultrasonic proved to be a rapid, non-destructive, and low cost technique for the measurement of different food characteristics [1–3]. Ultrasound may also be an alternative measurement method to discriminate types of flours for different purposes. The effect of ultrasound and sonication on some proteins in order to improve their functional properties has been studied [2–4]. Ultrasound treatment using 20 kHz probe may affect functional properties of proteins like solubility and foaming ability by sample exposure at high temperatures caused by sonication. Ultrasonic pretreatment gives also an increase in total sugar released and protein yield in a soy protein extraction when compared with non-sonicated samples [3]. The sonication-induced structural changes and thermal properties of proteins were investigated [5–9]. Thus, the enthalpy of penetration due to protein aggregation may increase. An ultrasound-assisted procedure for the extraction of defatted wheat germ proteins showed an increase from 37% to 57% as compared with non-treated samples [10]. The feasibility of using ultrasonic velocity measurement to assess the composition of some meat-based products was also demonstrated [11]. Other experiments on alkaline extraction of proteins from rice bran showed that the extraction time decreased, with increasing ultrasonic power [12,13]. Keeping in mind all this information, we tried to improve protein determination in cereals using a combination of a modified biuret protein assay and ultrasonic extraction procedure. Normally, the protein can be estimated from the nitrogen value. The Dumas method of protein analysis as well as the Micro-Kjeldahl procedure is standardized, being recommended for use in cereals [14,15]. The classical methods, like Micro-Kjeldahl procedure, require special equipment for mineralization and distillation, and where large numbers of samples are to be analyzed these steps are relatively slow. A colorimetric method based on the biuret reaction could be a procedure of choice because of its simplicity, the availability of equipment, and the potential to automate the method [16]. However, the classical biuret method

Abbreviations: DMF, defatted maize flours; UAE, ultrasound-assisted extraction; CV, coefficient of variation; R^2, the coefficient of determination; US, ultrasonic; N, normal; TSE, Traditional Solvent Extraction.

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cannot be directly applied to cereal flours due to the starch interference [17–19].

Therefore, in this work, an ultrasound-assisted procedure for the extraction and determination of cereal proteins was established. This investigation focused on the use of high-power ultrasound for fast extraction and determination of proteins from cereal seeds. Besides, another aim of our paper is quantifying zeins from various alcoholic extracts. Numerous combinations of reagents have been tested and the most suitable one for cereal protein determination was selected. Herein, we also demonstrate that the use of ethanol for preparing the biuret reagent and centrifugation may prevent the opalescence of supernatants and improve the protein determination.

2. Experimental

2.1. Materials

The reagents used in this work were of analytic purity and the solutions were prepared using deionized water (18.2 MΩ cm) from a Milli-Q system (Millipore, Bedford, MA). Zein from maize and bovine serum albumin (BSA) were from Sigma–Aldrich (Saint Louis, Missouri, USA) and used for analytical and comparative studies. Solvents used for extracting and degreasing zein such as ethanol, acetone, and petroleum ether were purchased from Merck (Germany), and used without further purification. Aqueous solutions of ethanol were prepared on a volume/volume basis and used for zein extraction. The alkaline–alcoholic solution (the biuret reagent) was prepared by solving 20 g of KOH in 100–200 mL of milliQ grade water, adding 450 mL of absolute ethanol or 475 mL of 95% ethanol (v/v), and completing with water to 1 L. We used maize kernels containing approximately 12% moisture from several Romanian and foreign maize hybrids and inbred lines taken from Suceava Plant Gene Bank, Romania. Both *opaque-2* and normal (non-*opaque-2*) maize varieties were analyzed. The kernels were ground and the resulted flour was defatted using petroleum ether as solvent in a Soxhlet extractor.

2.2. Instrumentation

Cereal seeds were ground in order to obtain maize flours using a commercial tripod portable mill (12 kg; 0.75 kW; 1410 rpm). Various particle-sized maize flours were screened with an analytical vibratory sieve shaker (Retsch, Germany). Coarse maize flour, which passes a certain sieve, for example the 710 μm one, was further milled to a fine powder using a laboratory electric cereal mill (SAMAP Mod F100, Andolsheim, France) with adjustable millstones. However, in order to characterize real maize seed samples, kernels were ground up to the desired granulation, the resulted flour fully passing a certain sieve (the best 100 μm mesh sieve). Test sieves with meshes from 1.6 mm to 100 μm were used. Ultrasonic extraction and the biuret reaction were performed on an ultrasound bath cleaner (J.P. Selecta Ultrasons system, 40 kHz; Barcelona, Spain). Comparative studies on zein extraction were made using a thermomixer (Thermomixer Compact Eppendorf AG 22331 Hamburg, Germany) or the eppendorf vials were just incubated at room temperature. Defatted maize flours (DMF) were obtained by Soxhlet Traditional Solvent Extraction (TSE) using petroleum ether as a solvent [20]. Zein extracts and the biuret clear solution of proteins were separated by centrifugation at 15,000 rpm using a Hettich Mikro 22R centrifuge (Tuttlingen, Germany). The NMR spectra were measured at 500.17 MHz for ^{1}H, and 125.78 MHz, respectively, for ^{13}C nuclei using a Bruker Avance III, 500 MHz spectrometer (Germany) equipped with a 5 mm PABBO detection probe. The NMR spectra were recorded in CD_3-OD, which is a suitable solvent for zeins. Number of scans for these experiments was 800 for ^{1}H and 10240 scans for ^{13}C nuclei. The zein extracts were analyzed by SDS–PAGE gel electrophoresis according to Laemmli protocol using a Mini-PROTEAN® Tetra Cell from Bio-Rad (Germany). The gels were visualized by Coomassie Brilliant Blue R_{250} staining procedure. For gel capture and analysis G: BOX F^3 gel scanner (Syngene, Germany) was employed. The absorption measurements were performed with a Biochrom Libra S35 PC UV–visible spectrophotometer (Cambridge, England) in quartz cuvettes of 10 mm (1 mL volume) in the range from 200 to 700 nm. Off-line nano-electrospray ionization (nanoESI) mass spectrometry was performed with the prepared samples using the Q-TOF II instrument (Waters MS-Technologies, Manchester, UK).

2.3. Procedures

The proposed assay of protein was tested on maize samples to determine the zein content of alcoholic extracts as well as the total protein (crude protein) of maize flours. Zein determination was investigated in view of further analysis of the nutritive quality of *opaque-2* and normal (non-*opaque-2*) maize. Both the extraction of zein using ethanolic solutions and that of total proteins by the alkaline–alcohol solution were investigated under ultrasonic conditions. Besides, ultrasonic stirring was applied to improve the biuret reaction in the presence of alcohol and insoluble copper phosphate. Ethanolic solutions reduce the opalescence of starch in maize samples, whereas the phosphate powder was the source of copper ions to be mobilized by the peptide bonds of proteins under alkaline conditions.

First, maize seeds were ground to get flours with different granulations. Then, samples of the resulted flours were degreased for 5 h with petroleum ether using the Soxhlet equipment.

For nanoESI measurements, samples of 5 μL of zein extracts in 65% and 95% alcohol were loaded into EconoTipTM emitters (ECONO10, New Objective Inc., Woburn, MA, USA) using Microloader pipette tips (Eppendorf, Hamburg, Germany). Experimentally, 5 μL of each sample were mixed with 1 μL of 37.5 mM ammonium hydrogen carbonate, pH 8.5, and incubated at 60 °C for 30 min. 4 μL of glacial acetic acid, pH 1.2, was then added to the solution and mixed prior to nanoESI measurement.

2.3.1. Zein extraction and determination

Zein from 50 to 200 mg defatted corn flour was extracted with 5–20 mL of 70% ethanolic solution under sonication or under normal shaking in a thermomixer (Eppendorf Thermomixer Compact, Eppendorf, USA) for 10–200 min. Maize flours with various diameter particles were sprinkled into eppendorf vials under stirring, onto 1 mL of ethanol solution which was previously pipetted in each vial. Zein was ultrasonic-assisted extracted in a batch mode using aqueous ethanolic solutions under the following conditions: temperature from 20 °C to 65 °C; extraction time 10–150 min; and solvent-to-solid ratio from 20 mL solvent: 1 gram flour to 2.5 mL solvent: 1 gram flour. Separately, zein extraction was performed under the same conditions except replacing the sonication with sample shaking in a thermomixer. After each extraction step, corn solids were separated from the corn-ethanol slurry by centrifugation at 15,000 rpm. The alcoholic extract (supernatant) was kept at room temperature in capped vessels until analyzed and/or further processed by evaporating and washing the zein precipitate. Herein, several experiments were performed on powder maize flour (<100 μm) and maize flour varieties that did not pass through 710 μm (>710), 500 μm, and 250 μm (>250) mesh sieves. Zein was extracted with different concentrated ethanolic solutions in the range from 60% to 96%. The most suitable extraction was performed using an 70% ethanolic solution.