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HPLC-Chip-Multiple Reaction Monitoring (MRM) method for the label-free absolute quantification of γ-conglutin in lupin: Proteotypic peptides and standard addition method

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ABSTRACT

In food science there is a growing demand of methods for the absolute quantification of proteins, such as allergens or bioactive proteins, and shotgun proteomics based on mass spectrometry is a promising analytical tool in this area. This paper describes an innovative label-free method for the absolute quantification of γ-conglutin, one of the most relevant lupin seed proteins, which is hypoglycaemic and a major allergen. The main features of the method are: (a) the chromatographic separation was performed on an HPLC-chip system coupled with an ion trap mass spectrometer; (b) five proteotypic peptides of γ-conglutin were selected and analysed with a multiple reaction monitoring (MRM) method; (c) absolute quantification was obtained by the standard addition method after purification of a reference sample of γ-conglutin from lupin seed; (d) the matrix effect was overcome by spiking with an exogenous protein, i.e. BSA, as internal standard.

1. Introduction

In food science, proteomics is applied to the assessment of the quality and safety of foods or food ingredients, having as a main objective the identification and quantification of bioactive proteins, in particular allergens and nutraceutics (Pischetsrieder & Baeuerlein, 2009). Proteins are used as bioactive ingredients in the formulation of dietary supplements or functional foods, i.e. foods that, beyond adequate nutritional properties, may improve the state of health or reduce the risk of some diseases. Some proteins of different origin are already used as nutraceutical ingredients or have been reported in the literature (Sirtori, Galli, Anderson, & Arnoldi, 2009).

Independently from their positive or negative activity, the quantification of food proteins is becoming a major issue in chemical analysis. Traditionally, target proteins are quantified by immunoenzymatic methods, which, however, have some drawbacks: (a) they rely on the time-consuming production and validation of specific antibodies; (b) some immunoassays are not sufficiently sensitive and may respond to a family of proteins rather than to a single target protein; (c) the lack of specific antigens may cause cross-reactivity problems and false positives (Murthy, Davis, Yatscoff, & Soldin, 1998); and (d) the possibility of adapting immunological methods to multiplex analysis remains limited (Rifai, Gillette, & Carr, 2006).

A powerful alternative to methods based on antibodies is provided by shotgun proteomics (Mamone, Picariello, Caira, Addeo, & Ferranti, 2009). The main absolute quantitative methodology is AQUA, which, however, is very expensive, requiring the individual synthesis, purification, and quantification of all isotope-labelled peptides spiked into sample: as a consequence, proteins are generally quantified using one single AQUA peptide (Barnidge, Goodmanson, Klee, & Muddiman, 2004; Fong & Norris, 2009), a fact that only rarely permit an accurate quantification. Other major limitations are the failure to take into account the actual proteolysis efficiency, since the labelled peptides are added after the tryptic digestion of sample, and the incompatibility with sample fractionation. Artificial concatamers of standard isotope-labelled peptides (QconCAT) (Beynon, Doherty, Pratt, & Gaskell, 2005) have been introduced as a smart strategy to achieve multiplex absolute quantification in one single experiment: although this has significantly improved the absolute quantitative measurement of proteins in biological samples, some limitations remain still unresolved, such as the high cost.

In recent years, increasing efforts have been directed towards label-free methods. Up-to-now, however, they have been successfully applied mostly to quantitative differential proteomics

Abbreviations: CL, calibration level; CL area, calibration level area; n-CL area, normalised calibration level area.

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(Brambilla, Resta, Isak, Zanotti, & Arnoldi, 2009), whereas applications to the absolute quantification of specific proteins are still rare.

The scope of this work was to develop an innovative HPLC-Chip-Multiple Reaction Monitoring (MRM) label-free method for the absolute quantification of γ-conglutin in complex samples, i.e. total protein extracts from lupin seed. Lupin protein is a food ingredient characterised by good nutritional value and technological flexibility that recently has attracted a lot of interest, since experimental and clinical investigations have indicated that it may be beneficial in the prevention of hypercholesterolaemia (Sirtori et al., 2004; Spielmann et al., 2007), hyperglycaemia (Magni et al., 2004), and hypertension (Lee et al., 2006). γ-Conglutin is an oligomeric protein, each monomer consisting of two subunits of 29 and 17 kDa linked by a disulphide bridge. These polypeptides derive from a single precursor of 46 kDa which is synthesised during seed development and processed by post-translational proteolysis. The large and small subunits originate from the N- and C-terminal regions of the precursor, respectively. The large subunit is glycosylated with covalently linked mannose and glucosamine units (Magni et al., 2004).

γ-Conglutin is a main bioactive lupin protein with very unusual characteristics, since it is simultaneously a hypoglycaemic agent (Magni et al., 2004) and an allergen (Magni et al., 2005). The most original features of our methodology are: (a) the translation of the "standard addition" method from the absolute quantification of small molecules (Ito & Tsukada, 2001; Maggi et al., 2009) to proteomics; (b) the use of a very specific and sensitive MRM method based on proteotypic peptides (Anderson & Hunter, 2006; Mallick et al., 2007); (c) the minimisation of the matrix effect by spiking with a known amount of an exogenous protein (internal standard); and (d) the very high chromatographic efficiency due to the use of a HPLC-chip-MS/MS system.

The "standard addition" method consists in comparing the curve of the reference analyte in a suitable solvent (reference curve) with the curve of the same analyte spiked into the samples where it must be quantified (in-matrix curve). This enables both the absolute quantification of the analyte, from the intercept of the in-matrix curve with the *X*-axis, and an estimation of the matrix effect, by comparing the slopes of the reference and in-matrix curves. In the presence of a negligible matrix effect, the two curves are parallel, whereas the slopes are divergent when the matrix impairs the analyte detection. By using this method and by spiking with an exogenous protein as internal standard (BSA) to minimise the matrix effect, it was possible to develop a reliable absolute quantification method by preparing a robust external calibration curve (normalised reference protein curve).

2. Materials and methods

2.1. Materials

Acetonitrile and formic acid for all uses were of HPLC quality (Baker, Deventer, The Netherlands); HPLC-grade water was prepared with a Milli-Q purification system (Millipore, Billerica, MA); sequencing-grade trypsin was from Promega (Madison, WI); BSA (>99% pure) and all other chemicals (reagent-grade) were from Sigma (St. Louis, MO).

2.2. Protein extract

The protein extract (LPE) from lupin seed (*Lupinus albus*) was obtained with a procedure developed in our laboratory (Brambilla et al., 2009). Briefly, lupin proteins were extracted from defatted flour with 100 mM Tris–HCl/0.5 M NaCl buffer (pH 8.2) for 2 h at room temperature. The solid residue was eliminated by centrifugation at 10,000 rpm, for 20 min at 4 °C, and the supernatant was dialysed against 30 mM Tris–HCl buffer (pH 8.2) for 24 h at 4 °C; the protein content was assessed according to Bradford (Bradford, 1976); dialysed solutions were stored at −80 °C.

2.3. Purification of reference γ-conglutin from lupin seed

Since a reference sample of γ-conglutin is not commercially available, it was necessary to purify it from a lupin protein extract (LPE) (Brambilla et al., 2009). This was done with two chromatographic steps, i.e. anion-exchange chromatography and gel-filtration chromatography. The protein extract was injected onto a DEAE-FF column (1.6 × 2.5 cm, 15–70 mm bead size, 5 mL column volume; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and fractions were eluted from the column with a linear salt gradient (0–100% NaCl over 16 column volumes). The γ-conglutin-enriched fraction, eluted at the beginning of the gradient (Supplementary Material Fig. S1A), was then loaded onto a gel-filtration column (Superdex 200) 1 × 30 cm, 24 mL column volume; GE Healthcare Bio-Sciences AB) for a second purification step (Supplementary Material Figure S1B). The column calibration was performed by a kit (GE Healthcare Bio-Sciences AB) containing ovalbumin, conalbumin, aldolase, ferritin, thyroglobulin and bluedextran. The molecular weight of purified γ-conglutin, calculated using the calibration curve ($y = -0.1394\ln(x) + 1.9809$; $r^2 = 0.9872$), agreed well with the expected molecular weight of γ-conglutin (Magni et al., 2007). Purified γ-conglutin was dialysed against 30 mM Tris–HCl buffer (pH 8.2) and stored at −80 °C. The concentration of the purified γ-conglutin was assessed according to Bradford (1976). This solution was used for the preparation of the calibration curves, since after freeze drying the resulting protein was partially insoluble.

2.4. Preparation of the quantification curves

The complete picture of the samples prepared for the quantification is shown in Table 1; in total four series of samples were analysed.

2.4.1. Reference curves

The two reference curves, i.e. the "reference protein curve" and "normalised reference protein curve", were built by analysing reference γ-conglutin without and with BSA as internal standard, respectively; increasing γ-conglutin amounts were digested in order to load on the HPLC-chip-MS/MS system 0, 5, 10, 20, 30, and 50 ng at each calibration level (CL) (Table 1A and C). The amount of BSA loaded on the chip at each CL in the normalised reference protein curve was 10 ng (Table 1C).

The samples for the "reference protein curve" were prepared by digesting increasing volumes of reference γ-conglutin with trypsin (0.5 mg/ml) in the ratio 1:50 enzyme/protein (w/w), after denaturation (urea 6 M), reduction (200 mM DTT), and alkylation (200 mM iodoacetamide) (Brambilla et al., 2009). The sample concentrations were reported in Table 1A. The samples for the "normalised reference protein curve" were prepared in the same way, but a constant amount of BSA was added to all samples before digestion; the final concentrations at each CL were reported in Table 1C. Each sample was analysed three times by injecting 2 μL.

2.4.2. In-matrix curves

The two in-matrix curves, i.e. the "in-matrix protein curve" and "normalised in-matrix protein curve", were prepared by spiking increasing amounts of reference γ-conglutin into a fixed amount of matrix, i.e. the LPE, containing all storage proteins (Brambilla et al., 2009), whose endogenous γ-conglutin content was unknown, without and with BSA as internal standard, respectively. The chip was loaded with 200 ng of the matrix and 0, 5, 10, 20, 30, or 50 ng of reference γ-conglutin at each CL (Table 1B and D).