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Identification and Quantification of ϵ-(γ-Glutamyl)lysine in Digests of Enzymatically Cross-Linked Leguminous Proteins by High-Performance Liquid Chromatography−Electrospray Ionization Mass Spectrometry (HPLC-ESI-MS)

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A rapid and convenient method for the precise quantification of ϵ-(γ-glutamyl)lysine isopeptide in lyophilized proteolytic digests of cross-linked plant protein samples was developed. The isopeptide was baseline-separated from three other isomers containing lysyl and glutamyl residues by reverse-phase high-performance liquid chromatography after exhaustive proteolytic digestion of the samples cross-linked by a microbial transglutaminase (MTG). Highly selective detection was performed by electrospray mass spectrometry in MS/MS mode. Demonstrating the applicability of the suggested analytical procedure, enzymatic cross-linking of protein isolates from soy [*Glycine max* (L.) Merr.], pea [*Pisum sativum* L.], and the sweet lupin species *Lupinus albus* L. and *Lupinus angustifolius* L. was investigated after incubation with 0.01 g of MTG/100 g of protein for 0−240 min at 40 °C. The liquid chromatography−mass spectrometry (LC−MS) method was successfully applied to monitor the kinetics of ϵ-(γ-glutamyl)lysine isopeptide formation. Since the calculated initial levels of ϵ-(γ-glutamyl)lysine in the genuine leguminous protein isolates were between 40 and 77 μmol/100 g, an isopeptide detection limit of 0.5 μg/mL, corresponding to approximately 50 μmol/100 g of protein, was shown to suffice for quantifying the cross-linking rate enzymatically induced by MTG. Concentrations of ϵ-(γ-glutamyl)lysine in the texturized proteins ranged from 100 to 500 μmol/100 g of protein.

KEYWORDS: **ϵ-(γ-glutamyl)lysine; transglutaminase; cross-link; leguminous proteins; LC−MS.**

INTRODUCTION

Microbial transglutaminase (MTG; EC 2.3.2.13) catalyzes the formation of ϵ-(γ-glutamyl)lysine cross-links (*1*). The resulting isopeptide bonds contribute to the formation of a stable protein network via intermolecular and intramolecular cross-links. As a consequence, a significant change in the functional properties of protein-containing foodstuffs, such as the textural improvement of sausages and ham and the manufacture of restructured meat, fish, and seafood products, is achieved (*2*). Further applications of MTG are known for milk and egg products as well as for noodles and pasta from cereals. Involved techno-functional changes include increases in viscosity and water-binding capacity, the formation of gel systems, and reduction of syneresis (*3*). Several reviews (*2*−*4*) and research papers (*5*−*8*) describe applications of MTG as an innovative tool in food processing. Consumers' demand for a greater variety of plant-based foods stimulated the research in developing new foodstuffs containing plant proteins.

It is anticipated that processing of protein isolates from a variety of plants, such as soy, wheat, pea, and sunflower, with MTG, modifying their technofunctional properties, will open up new areas of application (*9*). Furthermore, several research publications show high application potential for protein preparations derived from sweet lupins as a promising food ingredient concerning functional properties and nutritive value (*10*). Lupin flours with high protein contents as additives in bakery products (*11*, *12*) and the enrichment of fruit- and vegetable-based baby foods with lupin protein isolates with excellent organoleptic product properties (*13*) have been reported. Therefore the well-known protein isolates from soy (SPI) and pea (PPI) and, additionally, from two sweet lupin species (LPI) were selected for the present study on enzymatic cross-linking of plant proteins.

However, to texturize the product in the desired way, controlling of the reaction in the course of the cross-linking

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process is a prerequisite. For this purpose, the quantitative determination of the ϵ-(γ-glutamyl)lysine isopeptide bonds formed by the enzymatic reaction is essential.

Identification and determination of the ϵ-(γ-glutamyl)lysine isopeptide is traced far back in analytical chemistry (*14*). The analytical principle is based on an exhaustive proteolytic digestion of the sample prior to high-performance liquid chromatography (HPLC), with gradient elution on reverse-phase (RP) columns such as Zorbax C_8 (*15*, *16*), μBondapak C_{18} (*17*, *18*), Cosmosil AR C_{18} (*19*), or Ultrasphere ODS (*20*) at ambient or elevated temperatures (40−43 °C). Even though cation-exchange chromatography is widely accepted as a reliable method for quantification of ϵ-(γ-glutamyl)lysine (*14*, *21*−*23*), particularly by use of Ultrapac 8 columns in the lithium form in the latest studies (*20*, *24*), it has been more recently either replaced by the RP-HPLC techniques because of increased rapidity of analysis or used as an additional independent chromatographic method for unambiguous quantitative identification of ϵ-(γ-glutamyl)lysine (*15*, *16*, *20*). Focusing on RP-HPLC, reported sample preparation included precolumn derivatization either with *o*-phthaldialdehyde (OPA) for highly sensitive fluorimetric detection (*15*−*17*, *20*, *25*) or with phenyl isothiocyanate (PITC) for UV detection (*18*, *19*). To avoid coelution of large unknown peaks and interference of ϵ-(γ-glutamyl)lysine with other derivatives, precolumn derivatization had to be usually preceded by purification via preliminary chromatographic fractionation, which could be cation- (*17*) or anion-exchange chromatography (*20*), filtration on an Inertsil ODS-2 column (*16*, *25*), or even preliminary two-step separation on a cation-exchanger resin and a silica HPLC column (*18*). As an improved alternative without complicated preliminary separation, Sato et al. (*19*) proposed ultrafiltration cleanup of the digest and subsequent filtration on a Sep-Pak C_{18} cartridge prior to PITC derivatization and HPLC separation. Enhancing the methods of Griffin et al. (*15*) and Beninati et al. (*17*), the introduction of preliminary separation by reverse-phase HPLC and precise control of derivatization time and temperature by Sakamoto et al. (*16*) enabled the improved determination of ϵ-(γ-glutamyl)lysine cross-links and their distribution in foods. This methodical progress was highly appreciated, since a lack of precision in the known methods had been previously reported by several researchers (*15*, *18*).

Exhaustive proteolytic digestion of the samples is crucial in ϵ-(γ-glutamyl)lysine analysis, because incompleteness of hydrolysis would result in underestimation of ϵ-(γ-glutamyl)lysine isopeptide contents or even lead to the conclusion that the dipeptide is not present. The choice of the appropriate proteolytic system with broad substrate specificity in order to guarantee exhaustive decomposition of proteins was the subject of several investigations (*14*, *15*, *19*−*23*, *26*). Acid hydrolysis was shown to be unsuitable for the isolation of the isopeptide cross-link (*27*) but was used in comparison with enzymatically digested samples to demonstrate the completeness of enzymatic hydrolysis for effective isolation of the ϵ-(γ-glutamyl)lysine isopeptide (*28*, *19*) or was applied to the isolated dipeptide to verify its identity via the molar concentrations of glutamate and lysine of its hydrolyzate (*16*, *20*). On the basis of these studies, Sakamoto et al. (*16*) accomplished digestion of their food samples by sequential addition of the proteolytic enzymes Pronase (twice), leucine aminopeptidase and prolidase, again leucine aminopeptidase, and finally carboxypeptidase A. As one aim of the present study was to evaluate mass spectrometric techniques as a convenient, specific and fast detection method for ϵ-(γ-glutamyl)lysine directly in proteolytic digests of cross-linked samples, enzymatic digestion was performed according to this well-established method, which was already shown to be applicable to foods such as soybean flour, beans, and peas by Sakamoto et al. (*16*).

Sakamoto et al. (*16*) reported the distribution of isopeptide cross-links with a detection limit less than 1 μmol/100 g of protein, thus presenting a very sensitive and precise method for the determination of ϵ-(γ-glutamyl)lysine. However, due to the comparatively unspecific detection principle used in RP-HPLC analysis, ion-exchange HPLC and thin-layer chromatography (TLC) were necessary to confirm that it was ϵ-(γ-glutamyl)-lysine which was obtained with OPA analysis. To date among all methods based on proteolytic digestion and derivatization followed by HPLC separation and UV or fluorescence detection, the approach described by Sato et al. (*19*) is undoubtedly the most sensitive one.

Due to the large number of samples (50−100 per week) that were envisaged in the associated research work on enzymatic cross-linking of plant proteins by means of MTG, the development of a rapid method for the determination of ϵ-(γ-glutamyl)-lysine isopeptide was necessary.

Processed protein products derived from leguminous plants such as roasted soybean flour with 30−40% protein were shown to possess ϵ-(γ-glutamyl)lysine contents amounting to approximately 50 μmol/100 g (*16*). For plant protein isolates from soy, pea, and lupin containing more than 90% protein, contents of ϵ-(γ-glutamyl)lysine were expected to be even higher owing to the elevated protein concentration of the isolates. Moreover, after the application of MTG to protein isolate suspensions, the initial level of ϵ-(γ-glutamyl)lysine should be further increased. Therefore, a detection limit of approximately 50 μmol/100 g of protein was deemed to suffice for the analytical monitoring of the cross-linking reaction in model food systems based on plant proteins.

The aim of this work was to develop a simplified but precise and highly selective HPLC method, not requiring time-consuming sample preparation like preliminary chromatographic fractionation and derivatization. Applicability of the suggested analytical method should be demonstrated by investigations on the kinetics of the enzymatically induced formation of ϵ-(γ-glutamyl)lysine bonds in model food systems by use of four leguminous proteins. Gel strength analyses of the model systems before and after enzymatic cross-linking were intended as a simple tool to demonstrate cross-linking efficiency additionally by the technofunctional effect of transglutaminase activity to give further evidence of MTG activity in the samples under study.

MATERIALS AND METHODS

Protein Samples. Two commercial protein isolates, SUPRO Ex 33 (SPI; DuPont Protein Technologies International, N. V, Ieper, Belgium) and Pisane HD (PPI; Cosucra, Fontenoy, Belgium), from soy [*Glycine max* (L.) Merr.] and pea [*Pisum sativum* L.], respectively, were used as substrates for enzymatic cross-linking in addition to two lupin protein isolates produced on a pilot-plant scale. For the latter purpose, sweet lupin seeds from Grain Pool Pty Ltd., West Perth, Australia, were chosen because of their low alkaloid levels. Alkaloid contents, determined according to Wink et al. (*29*), ranged from 50 to 100 mg/kg in the seeds and were less than 10 mg/kg in the protein isolates, not exceeding the critical value of 200 mg/kg for lupin-based foodstuffs. The lupin protein isolates from seeds of *Lupinus albus* L. (LPI 1) and *Lupinus angustifolius* L. (LPI 2), respectively, were manufactured at the Fraunhofer Institute for Process Engineering and Packaging, Freising, Germany, by the patented process (*30*) for separation of fatty oil and removal of antinutritive components such as alkaloids and

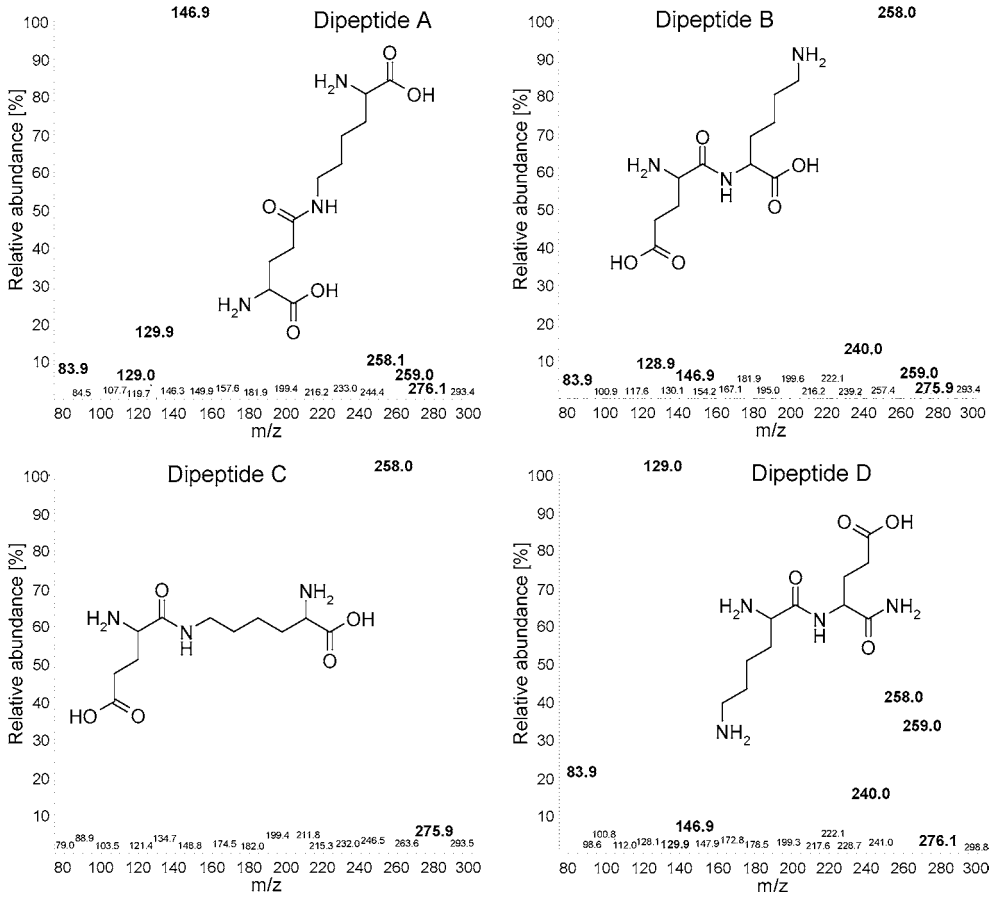

Figure 1. Chemical structures and mass spectra of dipeptides A [H-Glu-(H-Lys-OH)-OH = ϵ-(γ-glutamyl)lysine], B [H-Glu-Lys-OH], C [H-Lys(retro-Glu-H)-OH], and D [H-Lys-Glu-OH] containing lysyl and glutamyl residues.

soluble sugars, for example, verbascose or stachyose. Preparation of lupin protein isolates was performed via mild alkaline extraction (pH 7−8), followed by isoelectric point precipitation (pH 4−5) and spray drying (*30*). Protein contents were analyzed by means of the Dumas method (conversion factor $N \times 6.25$) (*31*) and ranged from 94% for LPI 1 to 92% for LPI 2. According to producers' data sheets, protein contents of the commercial protein isolates SPI and PPI were 90% ± 2%. Amino acid compositions of PPI and SPI are indicated according to producers' data sheets. For additional characterization of LPI 1 and LPI 2, Ansynth Service B. V., Roosendaal, The Netherlands, was charged with amino acid analysis of these protein isolates.

Chemicals. Dipeptide A [H-Glu-(H-Lys-OH)-OH = ϵ-(γ-glutamyl)-lysine] and its isomers B [H-Glu-Lys-OH], C [H-Lys(retro-Glu-H)-OH] and D [H-Lys-Glu-OH], also containing lysyl (Lys) and glutamyl (Glu) residues (**Figure 1**), were purchased from Bachem, Heidelberg, Germany. Boric acid, thymol (2-isopropyl-5-methylphenol), and trifluoroacetic acid (TFA) were from VWR International, Darmstadt, Germany. Water (HPLC grade) was obtained from Fluka, Buchs, Switzerland.

Enzymes. Microbial transglutaminase (81−135 units/g of enzyme preparation) derived from *Streptoverticillium mobaraense* was provided by Ajinomoto Co., Hamburg, Germany, as a powdery enzyme preparation consisting of 99% maltodextrin and 1% active enzyme protein.

Pronase (7.0 units/g of enzyme preparation) from *Streptomyces griseus*, in the form of a lyophilized powder, was acquired from Roche Diagnostics GmbH, Mannheim, Germany. An ammonium sulfate suspension of a leucine aminopeptidase (77−86 units/g of enzyme preparation) and a salt-free lyophilized powder of prolidase (194 units/g of enzyme preparation), both from porcine kidney, as well as an aqueous suspension of carboxypeptidase A (77−86 units/g of enzyme preparation) from bovine pancreas, were purchased from Sigma−Aldrich, St. Louis, MO.

Enzymatic Cross-Linking of Proteins. The cross-linking behavior of each protein isolate was studied in a simple model food system. Considering their potential in future applications as plant-protein based foodstuffs, such as a meat replacer with cut-stable appearance, the conditions for the cross-linking reaction were chosen. In preliminary experiments, the solubility and processability of the protein isolates were studied, particularly their ability to be stirred, providing a homogeneous protein suspension in water. According to the observed protein properties, suitable maximum concentrations in the cross-linking studies were 18%, 14%, 20%, and 13% for the used protein isolates from pea (PPI), soy (SPI), and lupin (LPI 1 and LPI 2), respectively. Cross-linked protein samples of 1000 g were produced by suspending the protein isolates in 2000 mL beakers for 30 min at 20 °C in 2% (w/w) sodium chloride, by use of an IKA Eurostar anchor stirrer (Staufen, Germany) at 200−400 rpm. An aqueous suspension of the MTG preparation was then added to achieve a transglutaminase/protein ratio of 0.01 g/100 g. Directly after mixing for 30 s at 20 °C by use of the anchor stirrer at 400 rpm, incubation was carried out in standard bloom test vessels (Schott, Mainz, Germany) in a water bath at 40 °C for 0−240 min. Samples were taken after 0, 30, 60, 120, and 240 min. The cross-linking reaction was stopped by inactivating the enzyme at 90 °C for 5 min. At the given protein concentrations and incubation conditions, the degree of cross-linking was expected to reach a maximum after 240 min according to the time-dependent saturation curves of gel firmness (data not shown). After freezing and storing the samples at −50 °C for 10−16 h, the samples were lyophilized for 48 h in a Beta 1-8 Christ freeze-dryer (Osterode/Harz, Germany), with the temperature not exceeding 25 °C during the supplementary drying. The ϵ-(γ-glutamyl)lysine isopeptide contents of the freeze-dried samples were analyzed by HPLC−ESI-MS after proteolytic digestion.

To control the efficiency of enzymatic cross-linking, gel firmness was measured with a TA XT plus/5 texture analyzer (Stable Micro Systems Ltd., Surrey, U.K.), with the SMS P/0-5 probe unit at 20 °C core temperature according to the AOAC official method for the determination of jelly strength of gelatin (*32*). Before and after

enzymatic cross-linking of the proteins, gel firmness of the respective sample systems was measured in duplicate.

Proteolytic Digestion. To obtain the ϵ-(γ-glutamyl)lysine dipeptide, proteolytic digestion of the cross-linked protein samples was performed according to the procedure suggested by Sakamoto et al. (*16*) and Kumazawa et al. (*25*), which was based on the methods of Griffin et al. (*15*) and Nonaka et al. (*26*). A 20 mg aliquot of the lyophilized cross-linked protein was dissolved in 3 mL of 0.1 m borate buffer (pH 8.0) after a small crystal of thymol was added. Proteolytic digestion of the preparation was carried out by sequential addition of proteolytic enzymes. Pronase was applied twice in dosages of 0.4 unit/mg of leguminous protein. Each incubation was performed for 24 h at 37 °C. The Pronase was then inactivated by heating the mixture at 100 °C for 10 min. In a second step, proteolytic digestion was continued for 24 h at 37 °C after addition of leucine aminopeptidase (0.4 unit/mg of leguminous protein) and prolidase (0.45 unit/mg of leguminous protein). After 24 h, leucine aminopeptidase (0.4 unit/mg of leguminous protein) was again added for another 24 h. In a third step, the preparation was digested with additional carboxypeptidase A (0.2 unit/mg of leguminous protein) for 24 h. For each proteolytic enzyme, the enzyme/leguminous protein ratio was adjusted to approximately 0.003 g/100 g. To inactivate the enzymes, the mixture was finally heated at 100 °C for 10 min. The proteolytic digest was lyophilized for 24 h, under the same conditions as described above for freeze-drying of the cross-linked protein samples.

LC−MS Determination of ϵ-(γ-Glutamyl)lysine. For LC−MS analysis, a Surveyor MS HPLC system was connected with a LCQ Deca ion trap mass spectrometer, both from Thermo Finnigan, San Jose, CA. HPLC separation of the peptides was achieved by isocratic elution with water containing 0.1% TFA at 15 °C and 0.6 mL/min, on a Hypersil ODS C_{18} column (250 × 4.6 mm i.d., 5 μm; CS Chromatographie Service, Langerwehe, Germany). MS detection of the dipeptides was based on electrospray ionization (ESI). For quantitative determination of ϵ-(γ-glutamyl)lysine (dipeptide A), the instrument was operated in the MS/MS mode, monitoring $m/z = 276$ to 147 and $m/z = 276$ to 130 transitions. For HPLC analysis, the total amount of each lyophilized digest was dissolved in 7.0 mL of demineralized water. An aliquot of 20 μL was injected directly after membrane filtration (0.45 μm). LC−MS analysis was performed in duplicate. To evaluate linearity, repeatability, and the detection limit of the LC−MS method, each calibration standard of the dilution series was injected 5-fold.

RESULTS AND DISCUSSION

Separation and Identification of Dipeptide Isomers. MS/MS detection was applied for the identification and quantification of ϵ-(γ-glutamyl)lysine in proteolytic digests in order to shorten previous HPLC approaches. In this way, further extensive sample treatment, such as fractionation or derivatization, and additional identification by ion-exchange HPLC and thin-layer chromatography (TLC) was avoided. By application of highly specific mass spectrometric detection of the ϵ-(γ-glutamyl)lysine isopeptide (A), interference with other dipeptides having a mass of $[M + H]^+ = 276$ was excluded. The mass spectra of related isomeric dipeptides containing lysyl and glutamyl residues are shown in **Figure 1**. In a first step, the retention times of the four dipeptides were determined. Under the conditions applied, baseline separation of the peptides A, B, C, and D was achieved (**Figure 2A**). Furthermore, in the MS/MS spectrum, isopeptide A [ϵ-(γ-glutamyl)lysine] is characterized by the fragments $m/z = 147$ and $m/z = 130$. While the first can be ascribed to the protonated lysine fragment, the latter can be attributed to the deaminated lysine fragment. In contrast, the elimination of water or ammonia yielded a typical fragmentation of the dipeptides B and C. For their distinction, the fragments displaying $m/z = 240$ and 128 (B) were suitable. The spectrum of isomer D was characterized by formation of the deaminated lysine fragment, yielding only traces of a lysine fragment. Thus, all four dipeptides could be unequivocally separated and assigned by their fragmentation patterns.

Table 1. Linearity and Detection Limit of ϵ-(γ-Glutamyl)lysine (Dipeptide A) Determination by HPLC–ESI-MS Analysis

dilution factor of std soln	ϵ-(γ-glutamyl)lysine in std solns (μg/mL)			
	expected	detected[a]	parent soln (calcd)	recovery (%)
1[b]	100.0	na[c]		
10	10.0	10.03 ± 0.18[d]	100	100
20	5.0	4.95 ± 0.19	99	99
50	2.0	1.96 ± 0.13	98	98
100	1.0	1.07 ± 0.07	107	107
200	0.5	0.50 ± 0.04	100	100

[a] Contents recalculated from the peak areas of the standard chromatograms and the linear calibration function between dipeptide A concentration (X in micrograms per milliliter) and signal intensity based on $m/z = 276$ to 147 and $m/z = 276$ to 130 transitions (Y). HPLC–MS injections per standard: $n = 5$. Linear calibration equation in the range of 0.5–10 μg of dipeptide A/mL: intercept $I = 365\,437$, slope $S = 7\,902\,912$, coefficient of determination $R^2 = 0.9987$. [b] Parent solution. [c] Not analyzed. [d] Mean ± single standard deviation.

Table 2. Recovery Rates of ϵ-(γ-Glutamyl)lysine (Dipeptide A) in Spiked Protein Isolates from Soy, Pea, and Lupin

sample	ϵ-(γ-glutamyl)lysine (μmol/100 g)			
	spiked	calcd	detected[a]	recovery (%)
SPI[b]	0		348 ± 18	
	87	435	429 ± 11	98
	241	589	656 ± 36	112
	469	818	827 ± 50	101
PPI[b]	0		493 ± 31	
	96	589	724 ± 27	123
	328	821	858 ± 37	105
	493	986	1110 ± 69	113
LPI1[b]	0		442 ± 1	
	76	518	522 ± 21	101
	188	630	742 ± 48	118
	360	802	950 ± 81	118

[a] Mean ± single standard deviation. Samples were digested and injected in duplicate. [b] Protein samples after 240 min of cross-linking.

Verification of the ϵ-(γ-Glutamyl)lysine Quantification. For calibration, standard solutions of ϵ-(γ-glutamyl)lysine (dipeptide A) in water were prepared by diluting a parent solution containing 100 μg/mL with dilution factors of 10, 20, 50, 100, and 200. A linear response was produced in the 0.5−10 μg/mL range (**Table 1**). The repeatability of the measurements was 7.3%, as determined from the maximum standard deviation of the peak areas obtained by multiple injections of standard solutions in the range of 0.5−10 μg/mL (**Table 1**). Coefficients of variation (CV) ranged from 1.8% to 7.3% in standard analyses. Described repeatability was consistent with the CV range resulting from the analyses of spiked samples after injection in duplicate (**Table 2**).

To determine the detection limit on the basis of the signal-to-noise ratio, measured signals from samples with known low concentrations of the analyte are usually compared with those of a blank sample, establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3:1 and 2:1 is generally considered acceptable for estimating the detection limit (*33*). To visualize both noise and signal levels in samples and calibration standards, the chromatograms of the sample LPI 1, which possessed the lowest genuine ϵ-(γ-glutamyl)lysine content among all four protein isolates studied (**Table 3**), are shown before and after 120 min of cross-linking in **Figure 2C**,**D**, in comparison to the chromatogram of the lowest ϵ-(γ-glutamyl)lysine standard used, which was at 0.5 μg of dipeptide A/mL (**Figure 2B**). Regarding cross-linking studies, **Figure 2C** presents the relevant sample

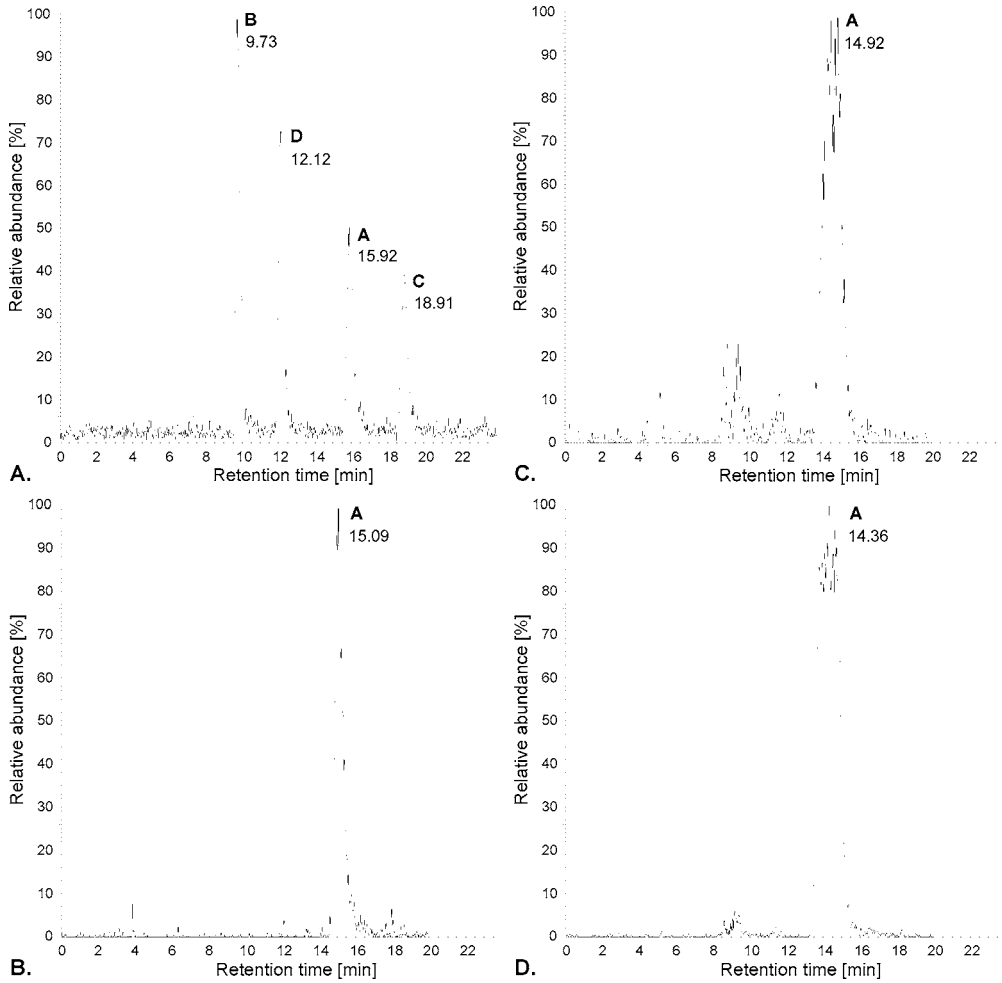

Figure 2. HPLC chromatograms of standards and digested protein samples in ϵ-(γ-glutamyl)lysine (dipeptide A) analysis: (panel A, upper left) Standard mixture of the dipeptide isomers A, B, C, and D (1.0 μg/mL) containing lysyl and glutamyl residues as specified in **Figure 1** (detection mode, MS full scan); (panel B, lower left) ϵ-(γ-glutamyl)lysine (dipeptide A) standard at 0.5 μg/mL; (panel C, upper right) sample LPI 1 after 0 min of cross-linking [40 μmol of ϵ-(γ-glutamyl)lysine (dipeptide A)/100 g]; (panel D, lower right) sample LPI 1 after 120 min of cross-linking [269 μmol of ϵ-(γ-glutamyl)lysine (dipeptide A)/100 g]. Detection mode in chromatograms B–D was MS/MS transition m/z = 276 to 147 and 130.

Table 3. Texturization of Protein Isolates from Soy, Pea, and Lupins: Comparison of Resulting ϵ-(γ-Glutamyl)lysine Contents and Corresponding Gel Strengths

	0 min cross-linking		120 min cross-linking	
sample	ϵ-(γ-glutamyl)lysine (μmol/100 g of protein)	gel strength (N/cm^2)	ϵ-(γ-glutamyl)lysine (μmol/100 g of protein)	gel strength (N/cm^2)
SPI	60 ± 3.6[a]	0.72 ± 0.14	227 ± 11	1.58 ± 0.21
PPI	77 ± 3.9	0.95 ± 0.16	333 ± 15	2.87 ± 0.33
LPI1	40 ± 2.6	0.13 ± 0.03	269 ± 13	0.34 ± 0.05
LPI2	45 ± 2.8	0.13 ± 0.03	483 ± 21	0.53 ± 0.07

[a] Mean ± single standard deviation.

blank, revealing a sufficiently high signal-to-noise ratio. On the basis of **Figure 2**, the detection limit of ϵ-(γ-glutamyl)lysine (dipeptide A) in aqueous solution was thus set to 0.5 μg/mL. By application of the conditions for digestion and sample preparation as described above, the concentration range of 0.5−10 μg/mL corresponded to approximately 50−1000 μmol of ϵ-(γ-glutamyl)lysine/100 g of leguminous protein. Potential interference of the analyte with the matrix was checked by adding the standards to sample solutions before proteolytic digestion and subsequent determination of recovery rates (**Table 2**). The amount of added ϵ-(γ-glutamyl)lysine to the matrix before digestion was 17−25%, 43−69%, and 81−135% (column 2 in **Table 2**) of the ϵ-(γ-glutamyl)lysine content in the corresponding unspiked sample. Calculated values for SPI, PPI, and LPI 1 (column 3) were compared with the corresponding detected contents. Resulting recovery rates (column 5) ranged between 98% and 123%.

Determination of ϵ-(γ-Glutamyl)lysine in Cross-Linked Plant Proteins. While the genuine ϵ-(γ-glutamyl)lysine contents of all four protein samples were clustered closely around 50 μmol/100 g protein, the concentrations of ϵ-(γ-glutamyl)lysine in the cross-linked plant proteins ranged from approximately 100 to 500 μmol/100 g protein (**Figure 3**). In a variety of 96 different foods, Sakamoto et al. (*16*) found ϵ-(γ-glutamyl)lysine concentrations from 0.2 to 135 μmol/100 g of protein. In the cited range, high levels were related to materials with transglutaminase activities. This is consistent with the minimum levels found after enzymatic cross-linking in the present study.

Several studies have revealed the existence of endogenous transglutaminase activities in several plant tissues such as chloroplasts, mitochondria, cell walls, and cytoplasm in *Arabidopsis* (*34*) and in further plant sources (*35*−*38*), for example, tobacco flower (*Nicotiana tabacum*), root and leaf tissue of the dicotyledonous pea (*Pisum sativum*) and broad bean (*Vicia faba*) and the monocotyledonous wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) (*20*), and in lupin (*39*). Therefore, ϵ-(γ-

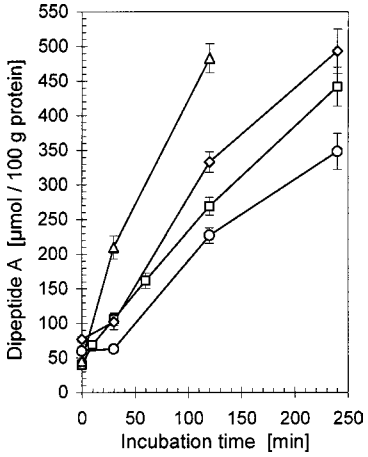

Figure 3. Texturization of protein isolates from soy (SPI), pea (PPI), and lupins (LPI 1 and LPI 2): resulting ϵ-(γ-glutamyl)lysine contents of the cross-linked protein substrates (dipeptide A). Contents of the protein isolates in the model food systems are given in parentheses: triangle, LPI 2 (13% w/w); rhomb, PPI (18% w/w); square, LPI 1 (20% w/w); circle, SPI (14% w/w). Error bars indicate a single standard deviation.

glutamyl)lysine could be endogenously generated in products of these plants as a posttranslational modification of proteins. Consistently with reports on increasing transglutaminase activities in roots and leaves of peas during growth (*20*), natural variations of the genuine content of ϵ-(γ-glutamyl)lysine found in plant protein products would be a consequence. This is supported by studies on the physiological role of plant transglutaminases in wound healing (*35*), senescence (*40*), and apoptosis (*41*). Additionally, transglutaminase activities of maize calluses and chloroplasts, being involved in initial differentiation, were light-sensitive and affected by hormone deprivation (*42*). High transglutaminase activities have been ascribed to rapidly proliferating and growing organs, with decreasing activities occurring during maturity (*38*). On the other hand, ϵ-(γ-glutamyl)lysine could be generated in plant protein-containing products during extraction and processing (spray drying), since it is formed as a consequence of heat treatment in reactions independent of transglutaminase (*16*, *43*). In the present study, processed leguminous protein isolates were used. Thus both ways for the formation of ϵ-(γ-glutamyl)lysine presumably contributed to the naturally occurring ϵ-(γ-glutamyl)lysine contents before the proteins were subjected to cross-linking with MTG.

By use of the proteolytic digestion for sample preparation described by Sakamoto et al. (*16*), the detection limit of our method was 0.5 µg of ϵ-(γ-glutamyl)lysine/mL in the digested sample solution (**Figure 2B**), corresponding to approximately 50 µmol/100 g of leguminous protein. Since the genuine ϵ-(γ-glutamyl)lysine contents of the plant protein isolates from pea, soy, and lupin used in this study were close to 50 µmol/100 g of protein (**Figures 2C** and **3**), the detection limit of the presented LC−MS method allowed analytical monitoring of the MTG cross-linking reaction. Compared to the HPLC method involving OPA derivatization (*16*), the newly developed LC−MS method provided a rapid and convenient analytical tool for the quantitative determination of ϵ-(γ-glutamyl)lysine cross-links in proteolytic digests of leguminous proteins treated with MTG for their textural modification.

Considering the large number of samples required for studying the kinetics of the cross-linking reaction, the accuracy of the method was deemed to be satisfactory. Calibration could cover ϵ-(γ-glutamyl)lysine amounts between approximately 50 and 1000 µmol/100 g of protein induced by cross-linking of plant proteins in texturized food systems. In the cross-linked plant protein samples investigated in this study, the maximum level recorded was 493 µmol of ϵ-(γ-glutamyl)lysine/100 g of protein (**Figure 3**). This level was in the range of the linear calibration. Baseline-separated peaks in HPLC chromatograms for ϵ-(γ-glutamyl)lysine in LPI 1 samples are shown in **Figure 2C** for an initial isopeptide content of 40 µmol/100 g of protein and in **Figure 2D** for the cross-linked sample after 120 min with 269 µmol/100 g of protein. Retention times for ϵ-(γ-glutamyl)lysine in cross-linked leguminous protein samples slightly differed from peaks for ϵ-(γ-glutamyl)lysine (dipeptide A) in the standard chromatograms (**Figure 2A,B**). This effect could be ascribed to matrix effects in the cross-linked protein samples during HPLC separation, because peaks were both clearly identified as ϵ-(γ-glutamyl)lysine by MS/MS detection and verified by spiking cross-linked samples (**Table 2**).

For the ϵ-(γ-glutamyl)lysine levels in the cross-linked protein samples, the corresponding protein contents were obviously not relevant in the studied range. After 30 and 120 min of incubation with MTG, the highest amounts of ϵ-(γ-glutamyl)lysine isopeptide were detected for a suspension of LPI 2, containing 13% protein, while the samples containing SPI, PPI, and LPI 1 at protein concentrations of 14%, 18%, and 20% showed clearly lower values (**Figure 3**). The amounts of resulting ϵ-(γ-glutamyl)lysine after MTG cross-linking rather seemed to be influenced by the source (plant species, harvest, growth, etc.) and the production process of the protein isolates (defatting conditions, temperature and pH during extraction and drying, etc.). A distinction in the quantity of available reaction sites in protein isolates from different sources for MTG cross-linking was evident. As the lupin protein isolate LPI 2 reacted faster than the other isolates, different steric availability of the reaction sites suggested itself. When the ϵ-(γ-glutamyl)lysine contents of enzymatically cross-linked leguminous protein isolates after incubation of the samples for 120 min with MTG and the corresponding gel strengths of the protein samples were compared (**Table 3**), the contribution of the absolute ϵ-(γ-glutamyl)lysine contents to the resulting gel strength could not be predicted and vice versa. For example, ϵ-(γ-glutamyl)lysine contents of only 227 and 333 µmol/100 g of protein for SPI and PPI resulted in high gel strength (1.58 and 2.87 N/cm^2, respectively) compared to ϵ-(γ-glutamyl)lysine contents of 269 and 483 µmol/100 g of protein for LPI 1 and LPI 2, which only formed weak gels with gel strengths of 0.34 and 0.53 N/cm^2, respectively. Hence, varying contributions of each reaction site in the three-dimensional structure of the substrate could be observed.

Furthermore, contents of both amino acids involved in the cross-linking reaction, that is, glutamine (19.1, 19.2, 20.6, and 21.2 g/100 g of protein for SPI, LPI 1, PPI, and LPI 2, respectively, all calculated as glutamic acid) and lysine (3.7, 3.8, 6.3, and 8.7 g/100 g of protein for LPI 2, LPI 1, SPI, and PPI, respectively), could not explain the observed gel strengths or isopeptide contents after cross-linking. For example, the high lysine contents in PPI and SPI resulted in the highest values measured for gel strength (**Table 3**), but their ϵ-(γ-glutamyl)-lysine contents were lower than those found in cross-linked LPI 1 and LPI 2, respectively. Data for cross-linked LPI 2 samples (lowest in lysine) showed highest isopeptide contents and weak gel formation, while cross-linked PPI (highest lysine content) resulted in lower isopeptide contents and the highest gel strength. Calculated relative amounts of lysine involved in the formation

of ε-(γ-glutamyl)lysine after 120 min of incubation with MTG were 0.5% for SPI and PPI, 1.0% for LPI 1, and 1.5% for LPI 2. The significant difference of these values in correlation with reaction rate, ε-(γ-glutamyl)lysine content (**Figure 3**), and resulting final gel strength (**Table 3**) confirmed varying availability of the reaction sites and contribution to texturization for the studied leguminous protein isolates.

This area of cross-linking leguminous proteins within the associated research project will be subjected to further studies, aimed at deeper insights into technological properties of enzymatically texturized plant proteins. In the present study, the controlling of the gel strength was a successful tool to demonstrate the efficiency of enzymatic cross-linking of leguminous proteins with MTG (**Table 3**). The new method for the determination of ε-(γ-glutamyl)lysine isopeptide contents could be used as a basis for further studies within the associated project and was anticipated to provide information about the correlation between enzymatic texturization by MTG cross-linking technology and gelation of leguminous proteins. For the four protein preparations, enzymatic cross-linking could be proven both chemically by the specific detection of the formed ε-(γ-glutamyl)lysine bonds and by the induced modification of functional properties in a model food system, that is, the increased gel strength (**Table 3**).

In conclusion, the present work describes a rapid and convenient HPLC method based on mass spectrometric detection in the MS/MS mode for the simultaneous identification and determination of ε-(γ-glutamyl)lysine in proteolytic digests of plant-protein based samples cross-linked with MTG. Due to the specific identification of the dipeptide in proteolytic digests via MS/MS fragmentation patterns, an additional thin-layer chromatographic identification of ε-(γ-glutamyl)lysine could be omitted. Furthermore, with the new method, complex fractionation of the samples and subsequent derivatization prior to HPLC analysis were no longer required. MS detection of ε-(γ-glutamyl)lysine in proteolytic digests can be regarded as an alternative approach for the quantification of this isopeptide in cross-linked leguminous proteins with contents of approximately 50−1000 μmol of ε-(γ-glutamyl)lysine/100 g of leguminous protein. For isolation of this dipeptide from the enzymatic digests via gel-filtration (*16*, *25*) and cation-exchange chromatography (*18*), established methods in combination with derivatization, for example, by use of PITC (*18*, *19*), OPA (*16*, *20*, *25*), dansylation (*14*), and fluorescence or UV detection, have to be considered as reliable techniques to estimate ε-(γ-glutamyl)-lysine contents, employing laboratory equipment readily available and providing results of isopeptide contents up to detection limits of 1 μmol/100 g of protein (*16*) or even as low as 3 nmol/g of protein (*24*). Another promising approach to determine ε-(γ-glutamyl)lysine with even lower detection limits than HPLC methods was recently reported by Sárvári et al. (*44*), applying a competitive enzyme-linked immunosorbent assay.

The presented LC−MS method is a significant improvement, since it provides simple sample cleanup and highly specific quantification and structural information, while facilitating at the same time high analytical throughput. Although the new method possesses lower sensitivity than established ones (*16*, *19*), it was shown to be suitable for monitoring the cross-linking reaction in simple model food systems, by use of two commercial protein isolates from soy and pea and two lupin protein isolates extracted at pilot-plant scale. This allows us to control the extent to which the proteins are cross-linked in texturization processes of foods based on leguminous proteins by applying transglutaminase technology for customized product development and modification of functional properties. Thus, strategies for specific product development of innovative food systems based on leguminous proteins can be designed.

ABBREVIATIONS USED

LPI, lupin protein isolate; PPI, pea protein isolate; SPI, soy protein isolate; MTG, microbial transglutaminase; OPA, *o*-phthaldialdehyde; PITC, phenyl isothiocyanate; TFA, trifluoroacetic acid; TLC, thin-layer chromatography.

SAFETY

Carboxypeptidase A from bovine pancreas is considered to be toxic if inhaled, contacted with the skin, or swallowed. When this enzyme is handled, safety precautions must be taken. A respirator, chemical-resistant gloves, safety goggles, and other protective clothing should be worn.

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