SZENT ISTVÁN UNIVERSITY

ANALYTICAL DETERMINATION OF BIOACTIVE PEPTIDES

Sándor Rapi

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Supervisor:

Prof. Péter Fodor DSc

Szent István University

Faculty of Food Science

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Name:	Ph.D. School of Food Science
Field:	Food Science
Head of School:	Gyula Vatai DSc Szent István University Faculty of Food Science Department of Food Engineering
Supervisor:	Prof. Péter Fodor DSc Szent István University Faculty of Food Science Department of Applied Chemistry

Signatures of the head of school and supervisor:

The applicant met the requirement of the Ph.D. regulations of the Szent István University and the thesis is accepted for the defence process.

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Head of School	Signature of Supervisor

1. INTRODUCTION

Small molecular weight peptides belong to an important family of compounds that play a major role in physiological processes (HARTMANN and MEISEL, 2007).
Their use in food science and human clinical areas is increasing; therefore it is important to study their properties in order to clarify the physiological role associated to these derivatives. Several publication have dealt with the separation, detection and identification of small molecular weight peptides in clinical and food science areas, that promote accurate knowledge of their effects.
The wide spectrum of biological effects make the low molecular weight peptide molecules promising functional food components, however, their safe use as food components requires the application of accurate, reliable and reproducible analytical methods.
The food applications are hindered in many cases because of low stability, but the elimination of stability problems may open the way to the usage as functional food additives.
The following methods can be used for the analysis of low molecular weight peptides, including separation (liquid chromatography, gel chromatography and size exclusion chromatography), detection (mass spectrometry) and their combinations. Modern and common determination methods require significant financial investment for acquisition and maintenance of the analytical instrumentation. A significant number of development laboratories, mainly linked to the food-processing industry, do not always have the state-of-the-art equipment necessary for the determination methodology,

therefore, these laboratories need reliable and reproducible methods that ensure the proper investigation and determination of the components. Accurate and precise analytical techniques provide opportunities for reliable determination of the peptide concentrations in different foods. In course of the research, the properties of low molecular weight peptides were investigated in order to perform analytical determination and stability characters with respect to their role in human biological areas and the food industry.

2. OBJECTIVES

During the research, properties of five small molecular weight peptides, circumstances of the development of reliable and sensitive chromatographic determination procedures, elimination of stability problems and food matrix effects have been determined as follows.

-Liquid chromatographic detection methods for the tested compounds.

-Stability test conditions for natural peptides, the use of protecting groups to eliminate potential stability problems (derivatization) or metal complexes formation.

-Impact of environmental factors on the quantity of the components (effect of the environmental temperature, additives).

-Possible effects of different food matrices and exploration of food components on peptide stability.

-Testing of antioxidant activity of selected peptides with FRAP method.

3. MATERIALS AND METHODS

3.1. Preliminary experiments for the quantification of free peptides

Determination of the UV-activity of the free peptide compounds was performed in the 190-400 nm wavelength range using spectrophotometry, and the maximum absorption wavelength of the peptides was determined.
Hereinafter, the free peptides were identified in the UV/VIS range by liquid chromatography using a diode array detector.
Due to the weak UV/VIS activity of small molecular weight peptides in the diode array detection, a parallel ELS detection was applied during the tests. Chromatographic tests were carried out using PL-ELS 2100 (USA) detector based on evaporative light scattering method on L-glutathione and γ-glutamyl-cysteine; HPLC separation of the peptide complexes with metal ions (Cd^{2+}, Zn^{2+}, Ag^{+}, Cu^{2+}) was also investigated.

3.2. Liquid chromatographic determination of small molecular weight peptides after derivatization

After the chromatographic tests of free peptides, derivatization was carried out for liquid chromatographic determination.
Peptides without thiol functional group were derivatized with Dansyl chloride and the detection was achieved with UV/VIS diode array detection, while thiol containing peptides

underwent OPA derivatization procedure and fluorescence detection method was used.
Quantitative studies were performed after the method development: the first step was the preparation of calibration solutions and signal integrals were determined.
External calibration was used for quantitative measurements, and a five-point calibration curve was fitted to the signal integrals as a function of the analytical concentration; furthermore, the detection limit values were determined as well.

3.3. Determination of the stability of various natural peptides depending on environmental conditions

Peptide standard solutions were studied under different conditions (pH, temperature effect, the impact of oxygen content of air and light radiation). Sample concentration was selected to 1000 mg/l in each case and these stock solutions were used to carry out the tests.
The following parameters were investigated: pH (pH=4.0, pH=7.0, pH=10.0) in three different media; temperature (-18 °C, + 4 °C; 30 °C); oxygen content (samples stored under paraffin layer in order to block oxygen dissolution and without paraffin), and light radiation (samples exposed to light and samples stored in the dark).

3.4. Examination of the stability of natural peptides with transition of metal complex formation

Stability assays of complex forming metals were carried out with three previously studied derivatives.
Stock solutions (20 mg/l) were prepared in two different pH ranges (pH = 4.8; 7.0) with three peptides (Apm, GSH, L-Car), while 1% starch solution was used for the carbohydrate matrix modeling studies.
The peptide solutions were stabilized with metal compounds (ferrous sulphate, zinc chloride, calcium chloride, magnesium sulphate) and the peptide concentration was determined in the test samples.

3.5. Determination of antioxidant activity of the investigated peptide derivatives with FRAP (Ferric Reducing Ability of Plasma) method

The FRAP experiments were carried out on peptide standard solutions. The standard peptide compounds were prepared at 100 mg/l concentrations and antioxidant activity of peptides was determined based on previously published methods (MAGALHAES et al, 2008).
Color intensity of the resulting complex was determined after five minutes of reaction time at 593 nm wavelength with absorbance measurement. The antioxidant activity values are given in ascorbic acid equivalents (AAE).

3.6. Determination of peptide content in food samples with the developed chromatographic methods

The selected samples were of crop (rice, peas, garlic) and animal (milk, cheese, sour cream, yogurt, kefir, cottage cheese) origin. According to the developed chromatographic methods, the thiol free peptides were derivatized with Dansyl chloride, while the OPA was used for the derivatization of peptides with thiol functionalities in accordance with the developed sample preparation method.

4. RESULTS

4.1. Results of the preliminary experiments

The examination of solutions resulted in UV/VIS absorption bands at 200 nm wavelength as low intensity signals.

Chromatographic analysis of the free peptides revealed that the chromatographic separation of the peaks was not appropriate, quantitation of the peptides was not feasible in this system.

Based on the conducted light scattering ELS detection methods, it can be concluded that determination of the thiol group containing peptides cannot be performed because the high detection temperature (about 100 °C) results in nebulization, evaporation and a high degree of degradation.

In the course of the stability studies, it was observed that the peptides connected to the metal ions in different ways: L-glutathione had three different complexes, while in case of γ-glutamyl-cysteine two different metal-peptide complex forms were confirmed by the mass spectrometry detection.

4.2. Results of liquid chromatographic determination of low molecular weight peptides after derivatization

These studies concluded that two different derivatization and detection methods should be implemented in case of low molecular weight peptide chromatographic

determination, according to the presence or absence of the thiol functional group in the tested peptides.
The detection of peptides without cysteine (Apm, Ala-Gln, L-Car) was appropriate with UV/VIS diode-array detector (290 nm) and Dansyl chloride as derivatization agent. It was found, that 10 µg/kg detection limit could be reached by using the developed method for the model peptides.
It was observed, that the Dansyl chloride derivatization of cysteine-containing peptides (GSH, γ-Glu-Cys) for liquid chromatography separation was less capable; in this case OPA derivatives were justified and the detection could be performed using a fluorescence detector at 336 nm excitation and 455 nm detection wavelength. The detection limit of the fluorescent detection method for sulfur-containing peptides could reach the concentration ranges of 1.0-2.0 µg/l.

4.3. Stability of natural peptides under various environmental conditions

Examination of the effects of environmental parameters on peptide stability revealed that the low molecular weight peptides were specifically sensitive to the effects of such environmental factors that commonly affect processed and raw food materials. Environmental pH (mainly low pH), oxidative conditions (longer contact with air) and light exposure (stored with longer sunlight radiation) can greatly damage peptide compounds; the stability is even lower when the test parameters collectively expose their effect.

4.4. Examinations of natural peptide stability by use of transition metals

Based on the research it was proved that metal complexes can increase the stability of the tested peptides.
The best protective effect was achieved by simultaneous presence of four metals used in the tests (zinc, calcium, magnesium, iron).
It was found that the used matrix influenced peptide stability independently of the metals present in the solution, because the protective properties of the metals were not effective when starch matrix was applied.

4.5. Antioxidant activity of the examined peptide derivatives

It can be concluded that significant antioxidant activity could not be detected with the method based on the iron reduction, so the antioxidant activity in the literature data is not essentially based on the radical scavenging ability that can be determined by the FRAP method.

4.6. Peptide content of the examined food samples

It can be concluded, that peptides did not occur in high quantities in the studied food raw materials and food products of plant and animal origin, and recovering these derivatives

would not be economically feasible; in addition, the peptides had a small degree of stability.

It was found that certain peptides occured in larger quantities in specific types of food. The γ-glutamyl-cysteine was found in larger quantities in the tested garlic and brown rice sample, while the amount of alanyl-glutamine was higher in pea samples compared to the other test samples.

5. NEW SCIENTIFIC RESULTS

1. It was determined during the liquid chromatographic determination of the test peptide compounds that, the model compounds (Aspartame, L-carnosine, L-glutathione, Alanyl-glutamine and γ-glutamyl-cysteine) cannot be separated in a single HPLC experiment with sufficient accuracy and reproducibility. Different functional groups represent different chemical characteristics and react differently with the same derivative forming reagent.
2. It was determined during the examination of peptides not containing cysteine moieties, that Dansyl chloride derivatization and UV (290 nm) diode array detection provides sufficient accuracy and precision to be implemented in quantitative experiments. The chromatographic separation was carried out on reversed phase chromatography column (Agilent Eclipse Plus RPC18 150X3, 3.3 mm). During the tests the detection limits for the model compounds were: Ala-Gln=17 µg/l, L-car= 16 µg/l, Apm= 160 µg/l.
3. During the examination of cysteine-containing peptides it was found that quantification can be carried out using a fluorescence detector (RF) after derivatives formation with OPA solution. The determination was carried out after derivatization; the injection was performed with 340 nm excitation and 455 nm detection. The chromatographic separation was carried out using a reversed phase column (Agilent Eclipse Plus RPC18 150X3, 3.3 mm). The detection limit (around 2 µg/l) was determined using the signal-to-noise ratio for both sulphur containing peptides,

respectively (GSH=2.1 µg/l, γ-Glu-Cys=1.3 µg/l), which is a significant improvement over previous methods.

4. In the course of the research it was determined that the stability of peptides was significantly different due to different environmental parameters. Decomposition of the peptides without cysteine moiety was substantial at acidic conditions (pH=4.0), at high temperatures (30 °C) and in the presence of oxygen, and the shelf stability was limited to several days. More intense level of degradation can be determined in case of peptides containing a thiol group relative to peptides not containing a thiol group due to the effect of the environmental parameters. As a result it was found that nearly 100% of the peptides were decomposed in a few days.
5. Based on the research it was proved that the formation of metal complexes increased the stability of the tested peptides. The best protective effect was measured for the combined use of four applied metals (zinc, calcium, magnesium, iron). However, it was found that the used matrix significantly affected the stability regardless of the metals, and the metal protecting effect could not be determined in a starch medium.
6. It was found that the studied plant and animal based food raw materials and finished products contained bioactive peptide test compounds in such a low amount, which would not allow their extraction in an economic way in view of their use as a food additive. Most food production processes (changes in the pH of the medium, mode and time of heat treatment) results in a reduction of the peptide concentration.

6. **RELATED PUBLICATIONS**

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