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Journal of Chromatography B, 846 (2007) 147–154
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Development of an at-line method for the identification of angiotensin-I inhibiting peptides in protein hydrolysates

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Received 10 May 2006; accepted 26 August 2006
Available online 25 September 2006

Abstract

A fast at-line method was developed for the identification of ACE inhibiting (ACEI) peptides in protein hydrolysates. The method consists of activity measurements of fractions collected from a two-dimensional HPLC fractionation of the peptide mixture followed by MS identification of the peptides in the inhibiting fractions. The inhibition assay is based on the inhibiting effect of ACEI peptides on the hydrolytic scission of the substrate Hippuric acid-His-Leu (HHL) during the ACE-catalysed hydrolysis reaction. A fast LC method was developed for the quantification of Hippuric acid (H) and Hippuric acid-Histidine-Leucine (HHL), allowing a large number of fractions to be analysed within a reasonable time period. The method is sensitive and uses only standard laboratory equipment. The limit of detection is 0.34 μM for the known ACEI peptide IPP. This is sufficiently sensitive for the identification of only moderately active peptides and/or ACEI peptides present at low concentrations. The relative standard deviation of the inhibition assay was 12% measured over a time period of 2 months. The IC50 value of IPP measured with the assay was 5.6 μM, which is comparable to the values of 5 μM and 5.15 μM reported in literature for the standard Matsui method. The assay was successfully applied in the identification of ACEI peptides in enzymatically hydrolysed caseinate samples. Two new, not earlier published ACEI peptides were identified; MAP (β-casein f102-104) and ITP (α-s2-casein f119-121) with IC50 values of 3.8 μM and 50 μM, respectively.

Keywords: ACE; Peptides; Assay; IPP; HPLC; MS; HHL; Hydrolysed caseinate

1. Introduction

Biologically active peptides play an important role in a large number of physiological processes in the human body such as immune stimulation, blood pressure regulation and cardiovascular functioning [1]. For the food industry peptides hence are attractive ingredients for the so-called functional-food products. In the past a variety of food-grade protein-containing sources, ranging from fish and meat to milk and maize have been investigated as potential sources for biologically active peptides [*e.g.* 2,3]. One of the fields that has been extensively studied is the use of small peptides to reduce hypertension [*e.g.* 4,5]. In the regulation of blood pressure certain specific, small peptides are known to inhibit the formation of the potent vasoconstrictor angiotensin-II (AII) from angiotensin-I (AI) by inhibiting the Angiotensin-Converting Enzyme (ACE). In order to get insight in the physiological behaviour of ACE inhibiting peptides, as well as to develop improved functional-food products, knowledge on the identity and generation of these peptides is crucial.

The identification of active peptides from complex natural ACE inhibiting food products or ingredients is a tedious and laborious task. Methods to do so generally rely on isolation of the active peptide(s) from the complex material using various sequential purification processes based on chromatography or electrophoresis, followed by identification of the peptides in the active fractions with *e.g.* Edman sequencing or mass spectrometry [*e.g.* 6]. Important aspects to consider when setting up an experiment aimed at the identification of active peptides

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doi:10.1016/j.jchromb.2006.08.052

in a complex sample include the selectivity and sensitivity of the activity assay used, as well as the substrate and reagents costs and the complexity of the required instrumentation. Moreover, the assay should preferably mimic the processes in the human body as closely as possible to minimise *in vitro* to *in vivo* differences. In order to improve the sensitivity and selectivity of the ACE assay many different substrates and quantification methods have been described. Popular substrates for the ACE assay include Hippuryl-Histidyl-Leucine (HHL) [7] and the internally quenched fluorescent substrate abz-FRK(dnp)P-OH [8] or *N*-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine (FAPGG) [9]. In our view the HHL substrate is attractive because it shows the closest resemblance to AI, the physiological substrate. Techniques to monitor the conversion rate of HHL include chromatographic or electrophoretic quantification of HHL or its products H and HL [10,11], or colorimetric or fluorimetric detection after OPA [12] or TNBS derivatisation [13]. Chromatographic quantification minimises errors due to background peptides but clearly is much slower than colorimetric methods. The best performance in terms of sensitivity and selectivity is obtained with the substrate abz-FRK(dnp)P-OH and fluorescence detection [9]. A potential drawback here is that the substrate is rather dissimilar from the physiological substrate AI.

All work aiming at the identification of ACE active ingredients published in literature so far is based on repeated off-line fractionation, activity measurement and identification [14–16]. A notable exception is the work by van Elswijk et al. [8]. In their work these authors apply an elegant on-line system consisting of a highly efficient HPLC separation, a quenched fluorescent substrate conversion assay and mass spectrometric identification. The ACEI peptides are separated by HPLC. A small part of the column effluent is directed towards the mass spectrometer for identification of the detected peptides whereas the major fraction is sent to a continuous flow ACE assay providing a continuous read-out of the ACEI activity. The method is sensitive and does not require laborious fractionation steps. Losses of peptides due to adsorption are minimised as no collection vials are used. The only drawback is that sophisticated dedicated equipment is required and again the dissimilarity of the substrate from AI.

In this paper we present a sensitive and selective at-line method for the identification of ACE inhibiting peptides in protein hydrolysates. High performance liquid chromatography followed by fraction collection is applied to fractionate the complex peptide mixtures. Activity measurements are based on the popular HHL-based Matsui assay as described by Cushman and Cheung [6]. Optimisation of this assay is discussed. The applicability of very fast LC-UV for quantification of the substrate conversion rate is demonstrated. Exact mass measurement is applied for the identification of the peptides in the fractions that show ACE inhibiting activity. Statistical information is given on the assay and the limitations of the method are discussed. Finally, the applicability of the assay is demonstrated by the identification of the peptides responsible for the ACE inhibition of an enzymatically hydrolysed caseinate.

2. Experimental

2.1. Materials and methods

2.1.1. Chemicals

Model peptides were obtained from different suppliers. The peptide IPP (purity >98%) was purchased from Bachum (Budendorf, Switzerland). The peptides MAP and ITP were synthesised at the University of Utrecht (Utrecht, the Netherlands). Angiotensin-I-converting-enzyme (ACE) and hippuric acid-histidine-leucine (HHL) were purchased from Sigma–Aldrich Chemie BV (Zwijndrecht, the Netherlands). All other chemicals were of high purity and were purchased from Merck (Amsterdam, the Netherlands). Enzymatically hydrolysed caseinate was kindly provided by DSM Food Specialties (Delft, the Netherlands).

2.1.2. Instrumentation

All peptide fractionations and identifications were performed on a Waters Alliance 2795 HT HPLC coupled to a Micromass QTOF-Ultima hybrid Time-of-Flight mass spectrometer equipped with the lock spray option for accurate mass determination (Waters, Almere, the Netherlands).

2.1.3. Analytical separation

Casein hydrolysate (10 mg) (DSM Food Specialties Delft, the Netherlands) was "dissolved" in 1 ml of water/acetonitrile 90/10 (v/v). The mixture was vortexed for 1 min, treated ultrasonically at room temperature for 30 min and was centrifuged at 14,000 RPM. The supernatant (50 μl) was injected onto an 150 mm × 4.6 mm Inertsil 5 ODS3 analytical column with a particle size of 5 μm (Varian, Middelburg, the Netherlands), equipped with a reversed phase C8 guard column (Waters, Etten-Leur, the Netherlands). Mobile phase A consisted of a 0.1% TFA solution in Milli-Q water. Mobile phase B consisted of a 0.1% TFA solution in acetonitrile. The initial eluent composition was 100% A. The eluent was kept at 100% A for 5 min. After this a linear gradient was started to 5% B in 10 min, followed by a linear gradient to 70% B in 40 min. Then a linear gradient was used to 90% B in 5 min and the eluent composition was kept at 90% B for another 5 min. Finally the eluent was reduced to 100% A in 1 min and the system was allowed to re-equilibrate for 14 min. The total run time was 80 min. The eluent flow rate was 1.0 ml min^{-1} and the column temperature was set at 60 °C. UV detection was performed at 215 nm and 254 nm. Ninenty percent of the eluent flow was split to the fraction collector, while the remaining 10% was introduced into the MS-source. The home-made splitter consisted of a zero dead volume metal T-peace (Valco Benelux, Nieuw Vennep, the Netherlands) of which one line was coupled to a restriction consisting of a 10 cm piece of 75 μm internal I.D. PEEK tubing coupled to the tubing of the fraction collector. The other line, consisting of 40 cm of the same PEEK tubing, was connected to the MS-probe. Fractions of 900 μl each were collected from 0 min to 65 min with interval times of 1 min in a polypropylene 1.4 ml 96-well plate (Micronic B.V., Lelystad, the Netherlands). The fractions were neutralized with 180 μl of a 0.15% aqueous ammonium hydroxide solution.

The solvent was evaporated to dryness under nitrogen at 40 °C using an Ultravap 96-well evaporation device (Porvair, Shepperton, UK). The residues were reconstituted in 80 μl of Milli-Q water and vortexed for 1 min. Then the content of each well was equally divided over two standard polypropylene 300 μl 96-well plates (Nunc, Roskilde, Denmark). The plates were stored at −20 °C until further use. One plate was used to determine the ACEI of the fractions. For active fractions the corresponding well of the other plate was used for further fractionation on a 150 mm × 2.1 mm Biosuite™ column with a particle size of 3 μm (Waters, Etten-Leur, the Netherlands). The separation was performed at 40 °C and a flow rate of 0.2 ml min^{-1} using mobile phase C consisting of a 0.1% formic acid (FA) solution in Milli-Q water and D consisting of 0.1% FA in methanol. The initial eluent composition was 100% C. A linear gradient was used to 70% D in 30 min, followed by a linear gradient to 90% B in 5 min. The eluent was reduced to 100% C in 1 min and the system was allowed to equilibrate for 14 min. The total run time was 50 min. From the Biosuite™ column fractions were collected with an interval time of 10 seconds in a polypropylene 300 μl 96-well plate. These fractions were than neutralized with 8 μl of the aqueous 0.15% ammonium hydroxide solution. After neutralisation the fractions were dried and reconstituted in 50 μl of Milli-Q water using the equipment and conditions described above. Forty microlitres of each fraction was pipetted into another 300 μl polypropylene 96-well plate which was used for the at-line assay. The plate with the remaining 10 μl of each fraction was used for structural identification of the peptides in the fractions showing ACEI activity. Both plates were stored at −20 °C until further use.

2.1.4. At-line Matsui assay

ACEI activities of the collected fractions were measured in a 96-well plate with chromatographic read-out. The initial conditions used for the assay were as published by Cushman and Cheung [6]. The optimum ratio of HHL and ACE, the incubation time and temperature as well as the reaction time and temperature were determined experimentally (see below).

2.1.5. Fast-LC analysis

The activity of the ACE enzyme was determined from the residual levels of the HHL substrate and the level of the product H formed. HHL and H were determined using fast-LC performed on a 25 mm × 4.6 mm Chromolith RP 18 Flash column (Merck, Darmstadt, Germany), equipped with a 10 × 4.6 guard column from the same supplier. The mobile phase composition, flow rate, and column temperature were optimised prior to starting the ACEI studies. In all experiments the injection volume was 30 μl. UV detection of HHL and H was performed at 280 nm.

2.1.6. Calculation of the percentage inhibition

The percentage inhibition of each fraction was calculated from the conversion of HHL to H relative to a blank measurement according to the following equation:

$$I\,(\%) = \frac{\mathrm{Cb} - \mathrm{Cf}}{\mathrm{Cb}} \times 100$$

In this equation Cb represents the percentage conversion of HHL to H for a blank water measurement and Cf the percentage conversion observed for a fraction. The percentage conversion was calculated according to the equation:

$$C\,(\%) = \frac{\mathrm{H}}{\mathrm{H} + \mathrm{HHL}} \times 100$$

Here H and HHL represent the measured peak areas of the two compounds.

2.1.7. Structural identification of ACEI peptides

For the identification of the peptides the remaining 10 μl fractions from the Biosuite™ column were diluted to 50 μl with an aqueous 0.1% TFA solution. Forty microlitres of each fraction was injected onto the LC–MS system. The separation was performed on the Biosuite™ column using the gradient described above. The source and desolvation temperatures were 100 °C and 300 °C, respectively. The cone and desolvation gas flows were 100 l h^{-1} and 800 l h^{-1}. The capillary voltage was 4 kV and the cone voltage 35 V. The collision energy was 10 eV. The collision gas was argon and the analyser pressure was 4e-5 mbar. In the MS–MS mode the collision voltage was increased to 25 eV. In the lock spray probe a mixture of PEG 300, PEG 600 and PEG 1000 (Sigma Chemicals, St Louis, USA) was used in a 10 mM ammonium acetate solution in methanol/water 1/1 (v/v) at concentrations of 1 μg ml^{-1}, 2 μg ml^{-1} and 4 μg ml^{-1}, respectively. The reference flow was 5 μl min^{-1}. Spectra were recorded at a resolution of 10,000.

3. Results and discussion

For the successful identification of ACEI peptides in complex mixtures the performance of the inhibition assay is crucial, i.e. it has to be very sensitive, fast and insensible to interferences. Evidently the actual performance of the assay strongly depends on the read-out method used to quantify the conversion of the substrate. Colorimetric or fluorometric methods are fast, but have a low sensitivity and the presence of interfering substances often results in high blank values. Chromatographic quantification of the substrate HHL and/or the H and HL formed can overcome these problems, but is much slower. Deployment of a very fast chromatographic method can reduce this disadvantage significantly.

3.1. Compatibility and miniaturization of the method

The determination of the ACEI activity of the chromatographic fractions is a crucial step in the identification of the actives. Before this measurement can be performed, the conditions of the fractions, *e.g.* pH and organic solvent level, have to be adjusted. In the current assay even low concentrations of organic solvents negatively influence the sensitivity. In the on-line ACEI method described by van Elswijk et al. [8], for example, the eluent is diluted using an inverse gradient to keep the methanol concentration at a constant level of 10%. The advantage of the at-line assay described in the present article is that neutralisation and solvent evaporation are carried out prior to the ACEI assay.

Due to this all types of organic modifiers and high modifier concentrations are allowed in the chromatographic fractionation step. Moreover, the sensitivity of the assay is positively influenced by the concentration step. The only disadvantage of course is that the procedure becomes slightly more laborious. By performing the fractionations and reactions in microtiter plate format and using well plate evaporators, the analysis time per sample can be reduced strongly.

3.2. ACEI assay

Optimisation of the Matsui ACEI assay was performed using a solution of the known inhibitor IPP in Milli-Q water at a concentration of 1 $\mu g\,g^{-1}$. Because the peptide concentrations in the HPLC fractions are relatively low, the ACEI assay should be as sensitive as possible. The performance of the assay in terms of sensitivity and speed strongly depends on the experimental conditions. The concentrations of the enzyme (ACE) and substrate (HHL), the incubation time and temperature as well as the reaction time and temperature were optimised starting from the values given by Cushman and Cheung [6]. The best results were obtained when 40 μl of the IPP solution was mixed with 25 μl of a 33.2 mU ml^{-1} ACE solution in phosphate buffered saline (PBS) pH 7.4 with a total chloride concentration of 400 mM. The optimum incubation time and temperature were 10 min and 37 °C, respectively. Variations in the ratio of ACE and HHL showed that the best results were obtained when 15 μl of a 0.35 mM HHL solution in PBS was added to each well. The highest percentage of conversion of HHL to H was reached when the mixture was allowed to react for 60 min at 50 °C. In order to stop the reaction the well was cooled in melting ice and stored at 4 °C prior to the fast-LC measurement. Using this ACE/HHL ratio, a conversion of HHL to H of 70% was obtained in the absence of inhibiting peptides.

3.3. Fast LC quantification of HHL and its conversion products

To keep the overall run time of the ACEI determination of the chromatographic fractions at an acceptable level, the run time of the chromatographic separation should be as short as possible. For instance in the procedure described by Meng et al. [9] the analysis time is 12 min, which would result in a total run time of 12 h when 60 fractions were to be analysed. In order to reduce the total run time a faster chromatographic method was developed. A generic route towards faster LC is the use of columns packed with smaller particles. A drawback of the use of this type of columns however, is the very high inlet pressure required. The recently introduced monolythic columns eliminate most of this drawback while still providing a very high separation speed. A Chromolith Flash RP18 column was tested for its ability to give a fast separation of HHL, HL and H. Various mobile phase compositions, temperatures and flow rates were tested with the aim to reach adequate separation of HHL and H from each other and from interfering UV adsorbing amino acids or peptides in the shortest possible time. HL does not respond at the wavelength used and hence does not interfere with the measurement. Gradient separations were not investigated as these will necessitate the use of column re-equilibration at the end of the run. The best compromise between separation and speed was obtained for a mobile phase consisting of 0.1% TFA in water/acetonitrile 85/15 (v/v) at a flow rate of 2 ml min^{-1} and a column temperature of 40 °C. The analysis time of each fraction using these conditions is 1.5 min, resulting in a total run time of 90 min when 60 fractions are to be analysed. The analysis time could even be reduced to 15 s when an eluent flow of 5 ml min^{-1} was used at a water/acetonitrile ratio of 90/10 (v/v). At these conditions, however, it is no longer possible to separate H from some UV-active amino acids and/or peptides present in some fractions.

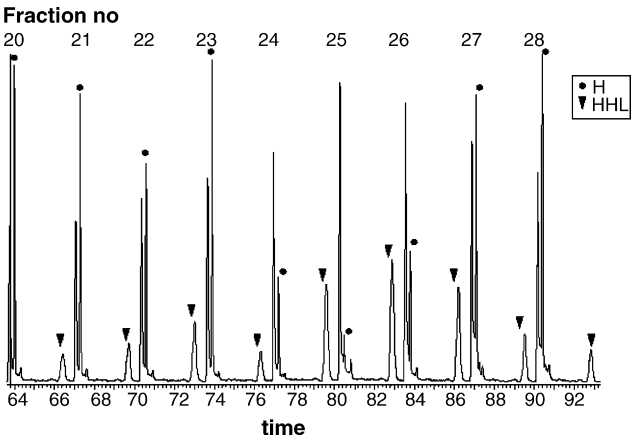

Fig. 1. Fast-LC chromatogram of nine subsequent activity measurements of HPLC-fractions of a hydrolysed caseinate.

A typical example of an ACEI measurement is given in Fig. 1. This figure shows the analysis of part of the fractions collected from the analytical ODS3 column after an injection of hydrolysed caseinate. The peaks representing H and HHL are marked with a circle and a triangle, respectively. Clearly the HHL/H peak intensity ratios vary and provide a measure for ACEI activity in the hydrolysed caseinate. In Fig. 2 the calculated inhibition versus the fraction number is graphically displayed. Obviously fractions 24–26 contain one or more ACEI compounds.

3.4. Assay sensitivity and reproducibility

In order to test the sensitivity of the at-line assay the inhibition of the known ACE inhibitor IPP was determined at concentra-

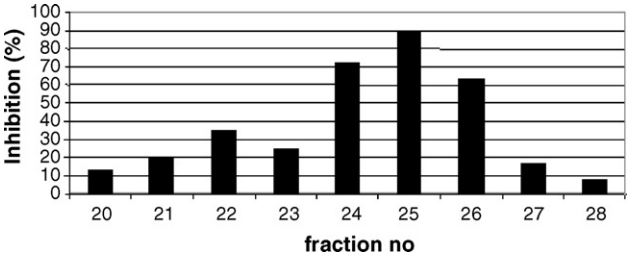

Fig. 2. Activity profile derived from the data shown in Fig. 1.

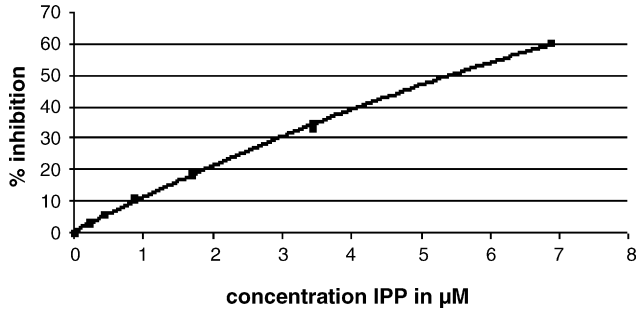

Fig. 3. Graphical display of the inhibition of IPP at different concentrations. Fitted line: $y = 9.508x^2 + 37.664x + 0.864$.

tions varying from 0 μg ml^{-1} to 2.24 μg ml^{-1}. Duplicate measurements were performed for each concentration. The results are graphically displayed in Fig. 3. The limit of detection of the assay was found to be approximately 0.43 μM for IPP. At this concentration the inhibition is 5.8% and well above the background noise of the measurement of 2%. The IC50 value of IPP was calculated to be 5.6 μM. This value is in good agreement with the values of 5 μM to 5.15 μM reported in literature [5,17]. For the determination of the reproducibility the IC50 value of IPP was measured 8 times covering a time period of 2 months. The averaged IC50 value was 5.6 μM with a standard deviation of approximately 12%.

3.5. Principle of the at-line assay

The principle of the full procedure for the identification of ACEI peptides is schematically displayed in Fig. 4. In order to get an impression of the distribution of the activity over the various peptides in the sample, the sample is first injected onto the ODS3 octadecyl modified column using the water/acetonitrile gradient. Fractions are collected in a 96-well plate with interval times of 1 min. Half of the volume of each fraction is pipetted into a second 96-well plate for further analysis on the Biosuite^{TM} column. The other half is than used to measure the ACEI activity for all fractions. Subsequently the fractions showing more than 5% inhibition are injected onto the Biosuite^{TM} column using the water/methanol gradient. Fractions are collected in a third 96-well plate now with an interval time of 10 s. Part of the volume of each fraction is pipetted into a fourth well plate. The third and fourth 96-well plates are used to create a more detailed activity profile and to determine the molecular ions of the peptides in the chromatographic peaks with increased activity. Finally MS–MS is used for the identification of the peptides in the active subfractions.

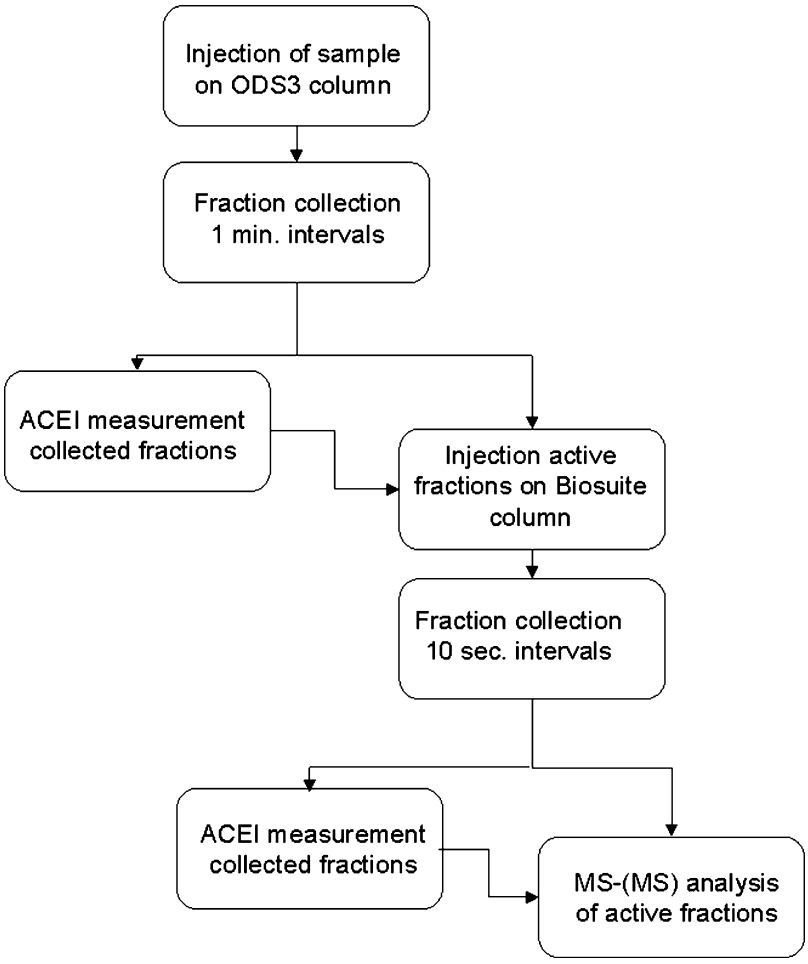

Fig. 4. Schematic principle of the at-line method for the determination of ACE Inhibiting peptides in complex mixtures.

3.6. Hydrolysed caseinate

In order to evaluate the applicability of the newly developed approach for real life samples, a hydrolysed caseinate was studied. Twenty microlitres of a 10 mg ml^{-1} sample solution was injected onto the analytical ODS3 column and analysed using HPLC-UV-MS. In Fig. 5a the UV trace at 215 nm and the MS-Total Ion Current (TIC) are given. The peptide distribution of the sample is obviously very complex. Many of the chromatographic peaks contain more than one ion, each representing a different peptide. In Fig. 5b the activity profile of the sample determined with the Matsui assay is given. The activity of the product clearly is distributed over a large number of peptides. In the region between 13 min and 20 min however, a clear increase can be seen. The full scanning MS analysis indicates that the activity of the fractions 15 to 17 can be explained from the presence of the known ACEI peptides IPP and LPP in the sample [22]. These two peptides, with IC50 values of 5 μM and 9 μM, respectively, are known to be present in hydrolysed caseinate [5,18]. In order to confirm this the hydrolysed caseinate was injected again, now collecting fractions between 13 min and 20 min with interval times of 10 s. The ACEI activity of each fraction was determined and the activity profile was compared to the MS-traces of the detected molecular ions. The result of the analysis is given in Fig. 6. For display purposes the peak height of the MS-traces was normalised to the measured activity of the peptide. This figure clearly shows that the profiles of the $[M+H]^+$ ion m/z 326.2 of IPP and LPP cover the activity of the peaks at 15.3 min and 16.7 min. The identity of the peptides that are responsible for the activity at 13 min to 14 min is unknown. The spectra of the fractions collected at 13 min and 14 min are very complex. As an example the spectrum of the fraction collected at 13 min is given in Fig. 7. The spectrum at 14 min contained largely the same ions, indicating that the peptides eluted partly in two fractions. A search of the most intense molecular ions present in the two fractions in a home-created

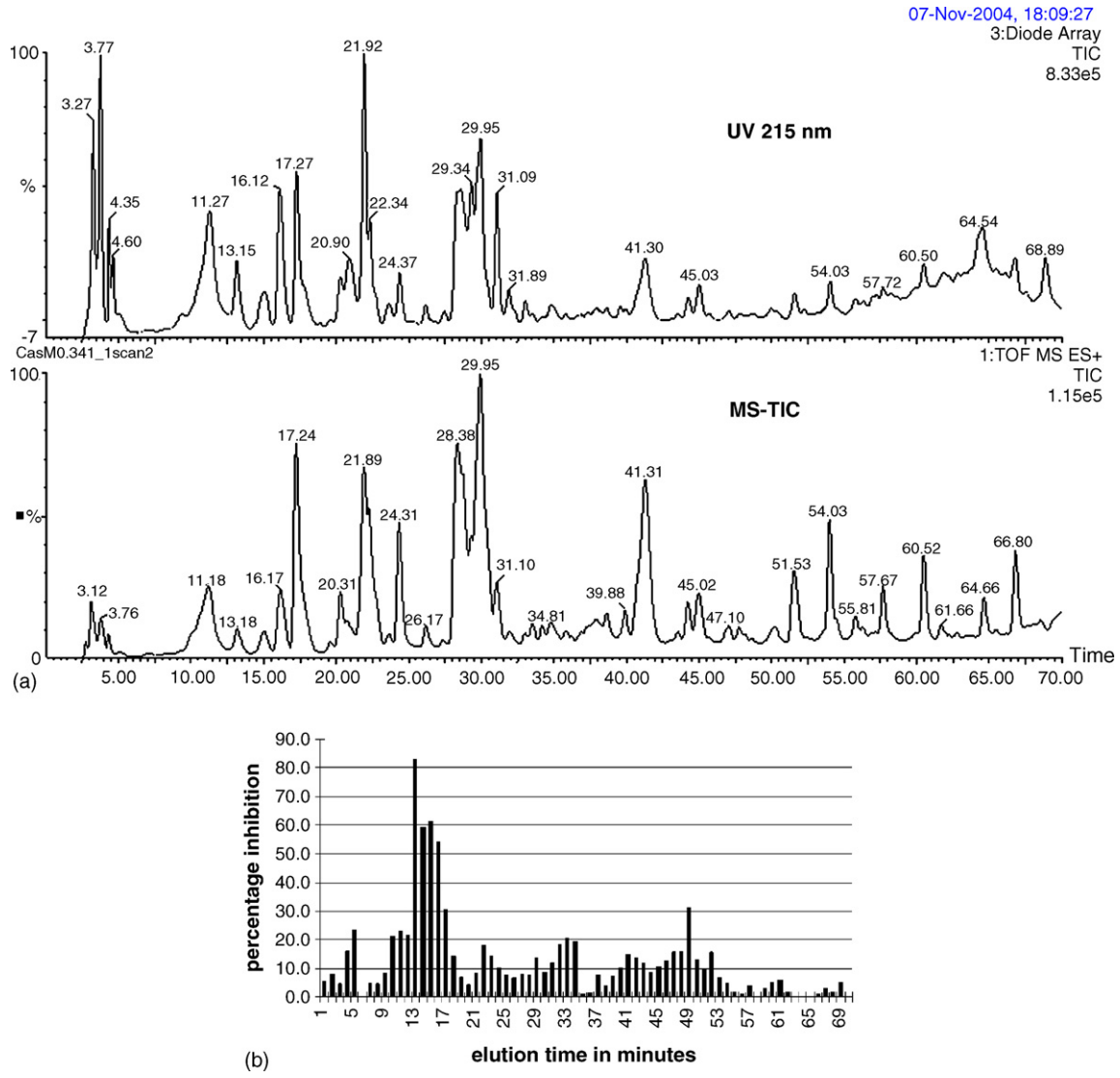

Fig. 5. (a) MS-TIC and UV (215 nm) chromatogram of a hydrolysed caseinate. (b) Activity profile of hydrolysed caseinate analysed on the ODS3 column (interval time = 1 min).

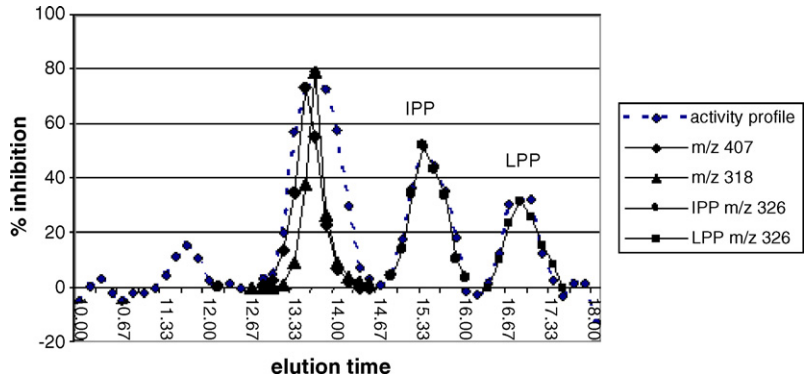

Fig. 6. Detailed activity profile and identified molecular ions of hydrolysed caseinate.

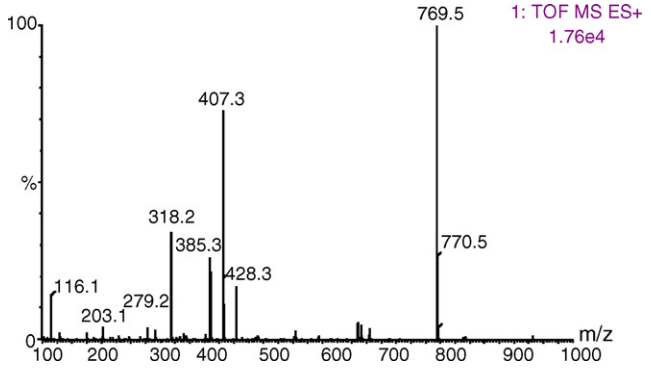

Fig. 7. MS spectrum of fraction 13 on the ODS3 column.

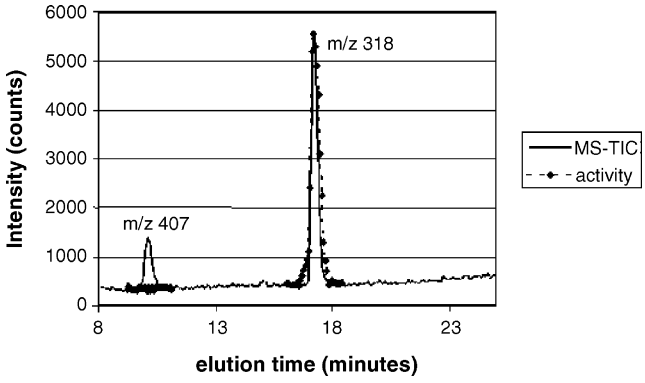

Fig. 8. Activity profile and MS-trace of the combined fractions 13 and 14 analysed on the Biosuite™ column (interval time = 10 s).

database of published ACEI peptides did not reveal the identity of the active peptides in these fractions. Only two peptides, with $[M+H]^+$ ions *m/z* 318 and *m/z* 407 from the spectrum of the fraction collected at 13 min match the activity profile of the peak eluting at 13.5 min.

To determine which of the two peptides was responsible for the ACEI activity measured at 13.5 min, the fractions 13 and 14 were combined and injected on the analytical Biosuite™ column. Fractions were again collected with interval times of 10 s. Each fraction was split and one part was used to measure the ACEI activity, while the other part was used for later identification of the peptides. In Fig. 8 the activity profile and the MS-TIC of fraction 13/14 are displayed. On the Biosuite™ column the compounds with the molecular ions *m/z* 407 and *m/z* 318 are now fully separated. The results clearly show that the peptide with the molecular ion *m/z* 318 is the source of the measured activity.

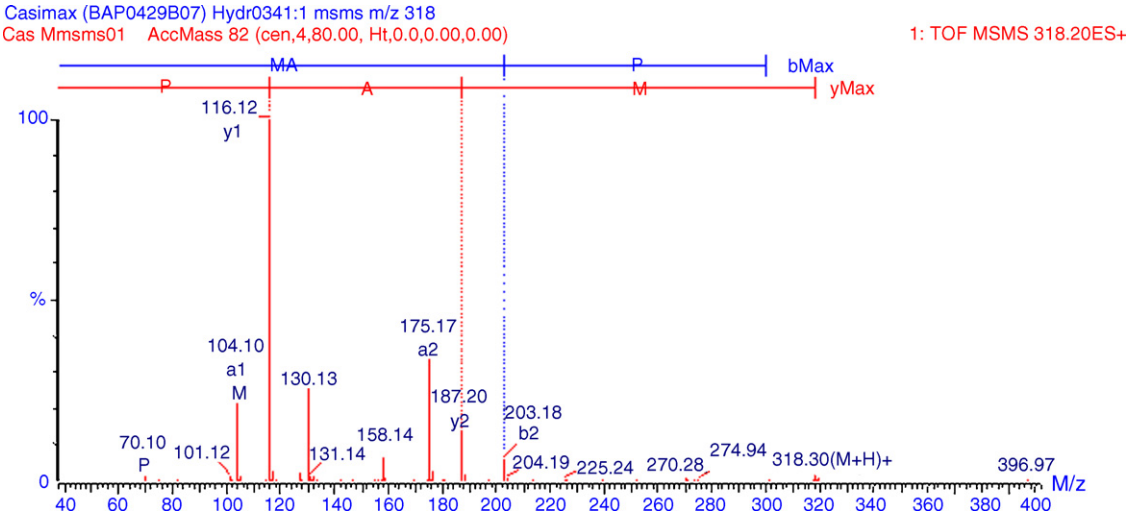

Fig. 9. Product-ion spectrum of *m/z* 318.

For the identification of *m/z* 318 the exact mass was determined and the product-ion MS–MS spectrum was recorded. The exact mass of the ion was found to be 318.1467 Da. A library search of this mass using a search window of 0.07 Da in the bovine proteins, α-s1-, α-s2-, β-, and κ-casein revealed only one hit, the tri-peptide Methionine–Alanine–Proline (MAP) originating from β-casein f102-104 with a mass error of −6.6 ppm. The MS–MS spectrum of *m/z* 318 was recorded and compared to the theoretical fragmentation pattern as described by Biemann and Roepstorff [19–21]. The MS–MS spectrum and the assigned fragments are given in Fig. 9. The exact mass measurement and MS–MS fragmentation pattern confirmed the assignment of the sequence MAP to the peptide with the molecular ion at 318 Da. Another new ACEI peptide, responsible for part of the activity at 15 min in Fig. 5b, is the peptide ITP originating from α-s2-casein f134-136. The measured $[M+H]^+$ ion *m/z* 330.2002 and the MS–MS spectrum confirmed the sequence of this peptide (not displayed). MAP and ITP were synthesised at the Utrecht University (Utrecht, the Netherlands). MS and MS–MS measurements confirmed the sequence of the two peptides. The peptides with the highest contribution to the total ACEI capacity of the sample were IPP (IC50: 5.6 μM), LPP (IC50: 9.6), MAP (IC50: 3.8 μM) and ITP (IC50: 50 μM). The peptides MAP and ITP are not previously reported and especially MAP is extremely potent.

4. Conclusions

A rapid at-line method was developed for the identification of ACEI peptides in complex mixtures. The assay consists of a 2-dimensional approach using two different RPC18 HPLC columns operated at different gradients. In the first analysis a rough picture of the activity profile is obtained, while in the second analysis fractions collected from the first column are analysed in detail on a different column. The assay can be performed using standard laboratory equipment. The sensitivity of the assay is 0.34 μM for IPP. This is sufficient to identify even those peptides that have only a minor contribution to the total activity of an ACEI product. The sensitivity is comparable or even slightly better than the value of 2 μM for IPP reported for the on-line method published by van Elswijk et al. [8]. In comparison to this on-line method the newly developed at-line method offers the advantage that different types of solvents can be used for the HPLC separation. Additional advantages are that there is no limitation to the percentage of organic modifier in the eluent and there is no loss of sensitivity due to dilution. The only drawback of our at-line method is the more laborious sample handling procedure.

The ACEI activity of the hydrolysed caseinate is distributed over a large number of peptides. Approximately 50% of the activity, however, is caused by a small group of peptides including the tri-peptides IPP and LPP. Two new, not earlier published ACEI peptides ITP (α-s2-casein f119-121) and MAP (β-casein f102-104) were identified using the at-line assay. The peptide ITP co-elutes with IPP and remained un-detected in single dimensional chromatographic approaches. The IC50 values of ITP and MAP are 50 μM and 3.8 μM, respectively.

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