Preparation of ingredients containing an ACE-inhibitory peptide by tryptic hydrolysis of whey protein concentrates


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Abstract

This study describes the characterisation of whey protein hydrolysates obtained from tryptic hydrolysis to assess their application as ingredients with angiotensin-converting-enzyme (ACE) inhibitory action. The levels of α-lactalbumin (α-la) and β-lactoglobulin (β-lg) remaining after hydrolysis were quantified. Peptides were separated by RP-HPLC, and Ala-Leu-Pro-Met-His-Ile-Arg (ALPMHIR), the most potent β-lg-derived ACE-inhibitory peptide was monitored. A correlation curve was established for the production of this peptide as a function of hydrolysis time. Heat-induced gelation of hydrolysates was studied by small-deformation rheology. The gelation times and the strength of the final gels were highly dependent on the degree of hydrolysis. Smaller peptides liberated by hydrolysis contributed to the inability of whey protein hydrolysates to gel.

Keywords: Whey protein concentrates; Trypsin hydrolysis; ACE-inhibitory peptides; Gelation

1. Introduction

Whey ingredients are used throughout the world in beverages, bars and other food systems. Newer whey ingredients include hydrolysed whey proteins that contain high levels of bioactive peptides (Foegeding, Davis, Doucet, & McGuffey, 2002; Gauthier & Pouliot, 2003; Meisel, 1998). These hydrolysates can be added to special foods to increase value, as enzymatic hydrolysis can optimise their functional properties (e.g., gelling, emulsifying and foaming capacities and solubility) (De Wit, 1998; Melachouris, 1984; Zydney, 1998). Thus, there is considerable commercial interest in the preparation of whey proteins for food, nutraceutical, and therapeutic applications (Torres, 2005).

Bioactive peptides are often inactive within the sequence of the parent protein and can be released, for example, by enzymatic hydrolysis with digestive enzymes. Bioactive peptide fragments originating from whey proteins should be taken into account as components that have a positive effect on cardiovascular health (Clare & Swaisgood, 2000; Pihlanto-Leppala, Koskinen, Piilola, Tupasela, & Korhonen, 2000; Seppo, Jauhiainen, Poussa, & Korpela, 2003; Yusuf, Lonn, Bosch, & Gerstein, 1999). For example, certain bioactive peptides may protect against hypertension through angiotensin-converting-enzyme (ACE)-inhibition and opioid-like activity, both in vitro and in animal experiments. The overall effect of an ACE inhibitor is the control of high blood pressure through dilation of blood vessels and its effect on blood volume (Belem, Gibbs, &
Lee, 1998; FitzGerald & Meisel, 2000; Masuda, Nakamura, & Takano, 1996; Mullally, Meisel, & FitzGerald, 1997a; Pihlanto-Leppala, Rokka, & Korhonen, 1998; Sipola et al., 2000; Walsh et al., 2004).

In recent research, ACE-inhibition fragments from both $\alpha$-lactalbumin ($\alpha$-la) and $\beta$-lactoglobulin ($\beta$-lg) were identified. It has been shown that ACE prefers substrates containing a hydrophobic (aromatic or branched side-chains) amino acid residue at the C-terminal position. Whey fractions hydrolysed with trypsin showed ACE-inhibition activity (Mullally, Meisel, & FitzGerald, 1997b).

ACE inhibition is measured by the concentration of substance needed to inhibit 50% of the original ACE activity (IC$_{50}$). A lower IC$_{50}$ value indicates higher efficacy. Published research studies on ACE inhibitory activity of various whey-derived peptides show results at a level of 77–1062 $\mu$M (Mullally et al., 1997a; Pihlanto-Leppala et al., 2000).

When developed as food ingredients, the processing of these antihypertensive peptides is vital to their activity. For example, heat treatments will have negative effects on the bioavailability of whey peptides, so processors must carefully monitor production parameters. Appropriate selection of enzymes for proteolysis will result in maximum biological activity and will limit the development of bitter flavour notes, with low cost. Trypsin is an interesting enzyme for the liberation of bioactive peptides, and also for the improvement of protein digestibility and for decreasing protein allergenicity, due to its specificity (Mullally et al., 1997b).

Additionally, it is important to study the functional properties of hydrolysates, because hydrolysis produced with different proteolytic enzymes can increase solubility and change gelling properties. Some enzymes can induce gelation following whey protein hydrolysis; others impair gelling properties (Doucet, Gauthier, & Foegeding, 2001; Foegeding et al., 2002; Otte, Lomholt, Ipsen, & Qvist, 2000). Gels confer structure, texture and stability to food products; they also allow the retention of large quantities of water and other small molecules inside the food matrix. These aspects are appreciated by processed food manufacturers.

Whey protein concentrates with $\sim$80% protein are frequently used to fortify various food products, owing to their low fat and lactose contents and high protein levels. This study describes the characterisation of hydrolysates of these whey protein concentrates produced using trypsin with different degrees of hydrolysis. For that purpose, whey protein concentrates were hydrolysed with trypsin to five different degrees of hydrolysis and the remaining levels of $\alpha$-la and $\beta$-lg were quantified. The resulting peptides were separated by reversed phase high-performance liquid chromatography (RP-HPLC), and the most potent $\beta$-lg-derived ACE inhibitory peptide, Ala-Leu-Pro-Met-His-Ile-Arg (ALPMHIR) reported to date (Mullally et al., 1997b), was identified and selected to characterize ACE-inhibitory activity of hydrolysates. Additionally, heat-induced gelation of three hydrolysates was studied by small-deformation rheology.

2. Experimental

2.1. Substrate and enzymes

A commercial spray-dried whey protein concentrate (WPC) from bovine milk with 80%, w/w, protein (WPC 80), Oragel HG80 from Armor Proteins (Saint Brice en Cogles, France) was used as substrate.

Trypsin from porcine pancreas with an activity of 1800 BAEE units mg$^{-1}$ of protein (1 U hydrolyses 1 $\mu$mol of N-$\alpha$-benzoyl-$\alpha$-arginine ethyl ester (BAEE) in a reaction volume of 3.2 mL and 1 cm light path at pH 7.6 at 25°C) was obtained from Sigma Chemical Co (St. Louis, MO, USA).

2.2. Hydrolysis of WPC 80

Solutions of WPC (prepared by suspending 2.5 g WPC in 50 mL of distilled water) were warmed to the hydrolysis temperature (37°C) and adjusted to pH 8.0 with 1 M NaOH. Enzymatic degradations were performed in a 0.05 L stirred, tank-type, batch reactor equipped with pH and temperature control. The pH was maintained at 8.0 with 0.25 mol L$^{-1}$ NaOH and the temperature was constant at 37°C. Trypsin was added at a concentration of 0.2 g L$^{-1}$, and the degree of hydrolysis (DH %) achieved was measured by the pH-stat method (Adler-Nissen, 1986). Five experiments were performed using the same initial conditions; the hydrolysis reaction was stopped at five different times (15, 30, 60, 120 and 180 min) by heating at 80°C for 5 min. Five resulting whey protein hydrolysates (WPH) were lyophilised using a CHRIST Alpha 2–4 system (B. Braun Biotech International, Osterode/Harz, Germany).

2.3. Physicochemical analysis

The moisture and ash contents were determined according to Food Chemical Codex (FCC, 1981a, b). Total protein content was determined according to the AOAC method (2000). Lactose analyses were carried out in an analytical HPLC unit (Jasco, Tokyo, Japan), equipped with a low-pressure quaternary pump (PU-1580), an evaporative light-scattering detector (LSD-Sedex 75, Alfortville, France) and a type 7125 Rheodyne Injector with a 10 $\mu$L loop. A Borwin Controller Software (JMBS Developments, Le Fontanil, France) was also used. The column was a Spherisorb NH$_2$, 5 $\mu$m, 250 mm length $\times$ 4.6 mm internal diameter (Waters Corporation, Massachusetts, Ireland). Elution was carried out with a mixture of 81% acetonitrile and 19% of water for 20 min. The flow-rate was 1 mL min$^{-1}$. The temperature of the heated drift tube was 45°C, the gas pressure was 3.0 bar, and gain 5. Sample preparation involved homogenisation of 200 mg of WPC80
or lyophilised hydrolysat with 10 mL of acetonitrile/water (40:60), followed by filtration with 0.45 μm Teknokroma (TR-200106-PVDF, 25 mm Ø, PK/100) syringe filters (Teknokroma, Madrid, Spain).

2.4. Chromatographic analysis of peptides and proteins

RP-HPLC was used for the separation of β-lg, α-la and the resulting peptides produced by hydrolysis. An analytical HPLC unit (Jasco) with a Chrompack P-300-RP column was used. Gradient elution was carried out with a mixture of two solvents (solvent A: 0.1% trifluoroacetic acid (TFA) in water and solvent B: 0.1% TFA in 80% aqueous acetonitrile, [v/v]). Proteins and peptides were eluted as follows: 0–1 min, 90% A; 1–10 min, 90–80% A; 10–15 min, 80–75% A; 15–20 min, 75–60% A; 20–30 min, 60–50% A; 30–33 min, 50–40% A; 33–36 min, 40–30% A; 36–39 min, 30–20% A; 39–41 min, 20–0% A. The flow-rate was 0.5 mL min⁻¹. The column was held at ambient temperature and detection was at 215 nm. Total run time was 50 min.

Prior to analysis by RP-HPLC, WPC and WPH were diluted with ultra-purified water, 0.5 g mL⁻¹ and 100 μL were injected. Standards of bovine β-lg and α-la were supplied by Sigma Chemical Co and dissolved in ultra-purified water that possessed a resistance greater than 15 MΩ. Under the conditions used, the major whey proteins, α-la and β-lg, were well separated and exhibited retention times of 34.5 and 37.5 min, respectively. The chromatographic system was calibrated by the external standard method with solutions that contained bovine α-la and β-lg, which were almost complete after 15 min. In contrast, hydrolysis of α-la was slower, and about 42% of the initial level of this protein was intact after 180 min of incubation. Concentrations of β-lg quantified by HPLC are presented in Table 1.

Peptides that eluted around 30% acetonitrile were collected and lyophilised for further sequencing by automated Edman degradation. Peptide sequence was determined by automated Edman degradation using an Applied Biosystem LC 491 Protein Sequencer (Foster City, USA), after chromatographic separation, collection and freeze-drying. The lyophilised ALPMHIR peptide was used as standard for quantification of this peptide in hydrolysates, using an external standard method.

2.5. Rheological measurements

The lyophilised WPH was first dispersed in 19 mM phosphate buffer (pH 7.0) to obtain a final concentration of 12%. Protein/hydrolysate concentration for the rheological experiments was limited by the solubility of the less hydrolysed WPH and gelation (even slight) of the most hydrolysed WPH. Hydration of proteins/peptides occurred at room temperature, over 3 h, under gentle stirring. Finally, the ionic strength of the medium was adjusted to 220 mM (expressed as NaCl concentration) by addition of NaCl.

Rheological measurements were performed with a controlled-stress rheometer AR2000 (TA Instruments, Delaware, USA) fitted with a rough acrylic plate geometry (40 mm diameter, gap 500 μm). The samples were covered with a thin layer of liquid paraffin to prevent evaporation, after having been placed in the measuring device. Each sample was submitted to a temperature ramp from 20 to 80 °C, at a rate of 2 °C min⁻¹; then, the temperature was maintained at 80 °C for 3.5 h. At the end of this time sweep, a frequency sweep (“mechanical spectrum”) was recorded over the range 0.06–62.83 rad s⁻¹. The sample was then cooled back to 20 °C, at the same constant rate (2 °C min⁻¹ yr, and held at 20 °C for 1 h. Finally, another frequency sweep (0.06–62.83 rad s⁻¹) was recorded at this temperature. During the temperature, time and frequency sweep measurements, a maximum shear strain of 1% was used. Temperature and time sweeps were performed at 6.28 rad s⁻¹.

3. Results

3.1. Characterization of WPC 80 Trypsin hydrolysates

The composition of WPC 80 and its WPH at five different degrees of hydrolysis (0.9, 2.2, 2.5, 2.8 and 4.2%) showed no significant differences between moisture, ash, protein, lactose and fat contents of the five WPH (p<0.01) using ANOVA. Mean values were 8.06±0.4%, 4.66±0.45%, 70.3±1.2%, 7.71±0.22% and 9.48±0.45%, respectively.

The disappearances of α-la, β-lg and the appearance of peptides were evaluated by HPLC (Fig. 1). Hydrolysis of α-la was almost complete after 15 min. In contrast, hydrolysis of β-lg was slower, and about 42% of the initial level of this protein was intact after 180 min of incubation. Concentrations of β-lg quantified by HPLC are presented in Table 1. In an earlier work performed using different experimental conditions, correlation curves for the hydrolysis of β-lg

![Fig. 1. Typical chromatogram of WPC and three tryptic hydrolysates (WPH0.9, WPH2.2, WPH4.2). (a) β-lg, (b) α-la, (c) the dotted box in the figure points out the elution of trypic peptides. *Peptide identified as ALPMHIR. Wavelength of absorbance was 215 nm. In the chromatograms for WPH 0.9, WPH 2.0 and WPH 4.2 the zero-time was successively shifted towards the right to show the changes in peaks throughout hydrolysis.](image-url)
with hydrolysis time were established (Mota et al., 2006). According to that study, the change in level of β-lg during hydrolysis was hyperbolic; the following equation was used for the model:

$$\beta\text{LG} = a \times t / (b + t),$$

(1)

where $a$ and $b$ are constants, $\beta\text{LG}$ is β-lg hydrolysis (% wt) and $t$ is hydrolysis time (in minutes).

In order to obtain these constants, the inverse of $\beta\text{LG}$ values were plotted against the inverse of time values so that a linear representation could be obtained and constants calculated:

$$\frac{1}{\beta\text{LG}} = \frac{b}{a} \times \frac{1}{t} + \frac{1}{a}.$$  
(2)

The linear functions obtained presented acceptable correlation coefficients. The correlation curve for hydrolysis of β-lg under the same conditions used in this work was:

$$\beta\text{LG} = 91.7t / (102 + t).$$  
(3)

A comparison between experimental and calculated (using Eq. (3)) concentrations of β-lg in WPC80 and its hydrolysates is presented in Table 1. The correlation between experimental and calculated data for hydrolysis of β-lg was 0.9995.

Hydrolysis with trypsin led to the production of a large number of peptides (Fig. 1). The average retention times for the seven major peaks, in order of increasing elution times, were 18.8, 22.4, 23.6, 25.9, 26.5, 29.4, and 31.7 min, respectively.

Mullally et al. (1997b) described that hydrolysis of β-lg with trypsin released ACE-inhibitory activity peptides eluting at around 30% acetonitrile, and the most potent peptide was f(142–148), with a charge-to-mass ratio of 837.05. This peptide had an IC$_{50}$ value of 42.6 μmol L$^{-1}$.

Much speculation has been presented as to the contribution of specific amino acid residues/sequences to ACE-inhibitory potency. Peptides containing hydrophobic amino acids at the three C-terminal positions are reported to be potent ACE-inhibitors (Cheung, Wang, Ondetti, Sabo, & Cushman, 1980; Saito, Wanezaki, Kawato, & Imayasu, 1994). Furthermore, structure-activity data suggest that a positive charge, as on the guanidine group of the C-terminal Arg, contributes substantially to the ACE-inhibitory potency of several peptides (Meisel, 1993). The sequence of the f(142–148) peptide, Ala-Leu-Pro-Met-His-Ile-Arg (ALPMHIR), reflects these observations. It is interesting to point out that the resistance of this tryptic peptide to further degradation by pepsin and chymotrypsin is controversial (Mullally et al., 1997b; Walsh et al., 2004). Mullally et al. (1997b) demonstrated that this peptide was essentially resistant to further degradation by pepsin and chymotrypsin but Walsh et al. (2004) demonstrated that synthetic ALPMHIR was rapidly degraded upon incubation with human serum.

The peptide f(142–148) does not have the potency of Captopril (a commercial drug inhibitor of ACE, with

<table>
<thead>
<tr>
<th>WPH</th>
<th>Time (min)</th>
<th>1/Time Conc β-lg (%a)</th>
<th>Hydrolysis β-lg (%a)</th>
<th>Concentration of ALPMHIR (% in freeze-dried product)a</th>
<th>1/Time Conc ALPMHIR (%)</th>
<th>Hydrolysis β-lg (%a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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<td>4.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
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</table>

*Results are expressed as % (w/w) of lyophilised.

Table 1

Concentration of β-lactoglobulin in WPC80 (time 0 min) and its hydrolysates (WPH 0.9, WPH 2.2, WPH 2.5, WPH 2.8, and WPH 4.2%) (quantified by HPLC. % β-lactoglobulin hydrolysis was calculated from experimental results and corresponding % of ALPMHIR peptide in lyophilised hydrolysate.
IC$_{50} = 0.006$ μmol L$^{-1}$. However, its IC$_{50}$ value is comparable with those found for other food-derived peptides with potential significant physiological effects as antihypertensive agents (Mullally et al., 1997b).

Edman sequencing revealed that the peptide with a retention time of 23.6 min from the chromatogram was ALPMHIR. Its concentrations in the five tryptic hydrolysates (WPH) are presented in Table 1. The extent of release of ALMPHIR, i.e., the actual amount detected and the theoretically expected amount from a known quantity of β-lg in the WPC starting substrate, were in good agreement.

A correlation curve was established for the production of the peptide ALPMHIR against hydrolysis time (Fig. 2). The linear function that enabled determination of equation curve were Eq. (4), and (5). The correlation between experimental and calculated data was 0.9968.

\[
\frac{1}{\%\text{ALPMHIR}} = 53.70 \frac{1}{t} + 0.631, \tag{4}
\]

\[
\%\text{ALPMHIR} = \frac{1.585t}{85.09 + t}. \tag{5}
\]

High hydrolysis times, >120 min, were required to guarantee that more than 50% of β-lg is hydrolysed and a significant amount of this peptide that is a potent inhibitor of ACE in vitro is formed. Concerning the antihypertensive potential of this peptide, it should be pointed out that it is dependent on its ability to be absorbed through the small intestine and to reach target organs without being degraded or inactivated by gastrointestinal or plasma proteinases and peptidases. Few studies in humans have been performed (Walsh et al., 2004).

3.2. Rheology

Whey proteins in aqueous dispersions, like other globular proteins, can form gels during heating if the protein concentration ($C$) is greater than the critical concentration for gelation ($C_0$) and the temperature ($T$) is greater than the denaturation temperature of the proteins ($T_d$) (Clark, 1992). Fig. 3 presents the behaviour of the elastic and the viscous moduli of three WPH dispersions during the thermal process applied. The initial dispersions had the same total protein concentration (17%, w/w), the ionic strength was 200 mM, expressed in NaCl concentration, and the pH was 7.0; they differed only in the degree of hydrolysis of each hydrolysate involved.

During thermal gelation the elastic modulus ($G'$) starts to increase and eventually crosses the viscous modulus ($G''$). Gelation time ($t_g$) for protein dispersions is the moment when $G = G''$ (Ikeda, Nishinari, & Foegeding, 2001), at $t_g$, only some protein aggregates are incorporated on the incipient initial three-dimensional network. From that moment, the sol fraction decreases with the incorporation of protein molecules or aggregates in the network (core of
the gel). The rheological parameters $G'$ and $G''$ start to increase at different rates, i.e. $\frac{dG'}{dt} > \frac{dG''}{dt}$, which reflects the increase in the elasticity of the gel; then, limit values are reached with $G' > G''$ (Clark, 1992; Gosal and Ross-Murphy, 2000).

After that, during the cooling process, $G'$ and $G''$ increase significantly due to the increase in non-covalent intra- and inter-molecular interactions, resulting in the loss of mobility of the proteins (Renkema, 2001). Spectroscopic studies reveal that hydrogen bonds mainly contribute to the hardening of the whey protein gels (Lefèvre and Subirade, 2000).

The gelation time (when $G' = G''$) is highly dependent on the degree of hydrolysis, as well as the strength of the final gels (compare final values of $G'$ for each gel), as depicted in Table 2. This behaviour can be explained mainly by the reduced amount of available intact protein for building structure. The stabilisation of the bigger peptides and proteins (components with tertiary structure) by smaller peptides liberated by hydrolysis has also to be considered. Huang, Catignani, and Swaisgood (1999) have reported this effect with the tryptic hydrolysis of a whey protein isolate and have attributed the loss of gelling ability to the stabilisation of $\beta$-lg (the main whey protein involved in the gelling phenomena) by a hydrophobic peptide liberated during tryptic hydrolysis.

4. Conclusions

ALPMHIR, the most potent $\beta$-lg derived ACE-inhibitory peptide, was assayed and a correlation curve was established for the formation of this peptide during hydrolysis with trypsin. Extensive hydrolysis was required to obtain peptides suitable for functional ingredients, namely ACE-inhibitory effect, more effective gastrointestinal absorption and reduced food allergies. However, smaller peptides liberated by hydrolysis lead to the inability of WPHs to form gel. Thus, the modulation of the gelling properties of whey proteins by enzymatic hydrolysis allows the incorporation of these ingredients, with ACE-inhibitory peptides, in liquid and semi-solid foods with high protein content (e.g., functional drinks with high protein content, dairy desserts, culinary sauces, etc.).

More studies are needed to establish the antihypertensive potential of this peptide in human beings, since this effect is dependent on the ability of this peptide to be absorbed through the small intestine and to reach target organs without being degraded or inactivated by gastrointestinal or plasma proteinases and peptidases.

References


AOAC. (2000). 33.5.03 AOAC Official Method 930.29. Protein in dried milk. AOAC International, Gaithersburg, MD, USA.


Table 2

<table>
<thead>
<tr>
<th>WPH</th>
<th>$t_g$ (h)</th>
<th>Final gel (after 5.5h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$G^*$ (kPa)</td>
<td>$G''$ (kPa)</td>
</tr>
<tr>
<td>0.9</td>
<td>0.4$^a$</td>
<td>12.0</td>
</tr>
<tr>
<td>2.2</td>
<td>0.7$^b$</td>
<td>2.8</td>
</tr>
<tr>
<td>4.2</td>
<td>2.9$^b$</td>
<td>0.05</td>
</tr>
</tbody>
</table>

$^a$t$_g$ occurs during heating step (20–80 °C), $^b$t$_g$ occurs during kinetics (80 °C).}

486


