Identification of a novel angiotensin-I-converting enzyme inhibitory peptide corresponding to a tryptic fragment of bovine β-lactoglobulin

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Abstract The angiotensin-I-converting enzyme (ACE) inhibitory activity of a tryptic digest of bovine β-lactoglobulin (β-lg) was investigated. Intact β-lg essentially did not inhibit ACE while the tryptic digest gave an 84.3% inhibition of ACE. Peptide material eluting between 20 and 25% acetonitrile during C_{18} solid-phase extraction of the β-lg tryptic digest inhibited ACE by 93.6%. This solid-phase extraction fraction was shown by mass spectroscopy to contain β-lg f(142–148). This peptide had an ACE IC_{50} value of 42.6 μmol/l. The peptide was resistant to further digestion with pepsin and was hydrolysed to a very low extent with chymotrypsin. The contribution of specific amino acid residues within the peptide to ACE inhibitory activity and the potential application of this peptide as a nutraceutical is discussed.

Key words: β-Lactoglobulin; Bioactive peptide; Gastric digestion; Pancreatic digestion; Nutraceutical

1. Introduction

Bioactive peptides which function as potent physiological regulators can be released during in vivo and in vitro hydrolysis of milk proteins by gastric and/or pancreatic enzymes [1]. Peptides which inhibit angiotensin-I-converting enzyme (ACE) have been isolated from many different food sources [2]. ACE (peptidyldipeptide hydrolase EC 3.4.15.1) has been classically associated with the renin–angiotensin system regulating peripheral blood pressure. The enzyme can raise blood pressure by converting angiotensin I to the potent vasoconstrictor angiotensin II. ACE is a multifunctional enzyme which also catalyses the degradation of bradykinin, and enkephalins [3]. Consequently, ACE inhibitory peptides may exert antihypertensive and immunostimulating effects and may increase neurotransmitter activity.

Several ACE inhibitory peptides have been isolated from enzymatic casein hydrolysates [1,2,4]. Only limited studies have been carried out on whey protein-derived ACE inhibitors. Albutensin A, a peptide derived from serum albumin, was shown to inhibit ACE [5]. Synthetic di- and tetra-peptides corresponding to α-lactalbumin (α-la) and β-lactoglobulin (β-lg) sequences were recently shown to inhibit ACE [6].

This study describes the isolation and characterisation of the most potent β-lg-derived ACE inhibitory peptide reported to date.

2. Materials and methods

2.1. Enzymes and substrates

Bovine trypsin (TPCK-treated, EC 3.4.21.4 Type XIII, 12700 BAEE U/mg protein), bovine chymotrypsin (TLCK-treated, EC 3.4.21.1 Type II, 40–60 BTEE U/mg protein), porcine pepsin A (EC 3.4.23.1, 3200–4500 U/mg protein) and ACE (EC 3.4.15.1, from rabbit lung tissue) were obtained from Sigma Chemical Co. Poole, Dorset, UK.

An enriched fraction of β-lg (89.8% (w/w) β-lg) was received as a gift from Dr. R. Mehra [7].

The β-lg peptide, Ala–Leu–Pro–Met–His–Ile–Arg corresponding to f(142–148) was synthesised using Fmoc solid-phase synthesis at BioResearch Ireland, University College Cork, Ireland. Plasma desorption mass spectroscopy (PDMS), which was carried out at the Biochemistry Department, University of Nottingham, UK, was used to determine peptide masses and to confirm peptide purity.

Hippuryl-L-histidyl-L-leucine and Captopril were from Sigma Chemical Co. Poole, Dorset, UK.

All other reagents were of analytical grade unless otherwise specified.

2.2. Laboratory scale hydrolysis of β-lg with trypsin

A 25 ml solution of the β-lg substrate, 8% (w/v) protein, was hydrolysed with trypsin at 50°C and pH 8.0 for 240 min. The pH was maintained constant using a pH-stat (Metrohm Ltd., Herisau, Switzerland). Following hydrolysis, trypsin was inactivated by heating at 80°C for 20 min, the hydrolysate was then cooled and stored at −20°C for further analysis. Details for the calculation of degree of hydrolysis (DH) were given previously [8]. The enzyme/substrate ratio (E:S), 0.003, was calculated on the basis of total protein content in the enzyme and substrate.

2.3. Treatment of β-lg f(142–148) with pepsin and chymotrypsin

The synthetic β-lg peptide corresponding to residues 142–148, i.e. Ala–Leu–Pro–Met–His–Ile–Arg, was incubated separately with chymotrypsin and pepsin. For each reaction, 500 μl of enzymatic solution (0.1 mg/ml) was added to 500 μl of peptide solution (6 mg/ml), mixed and incubated at 50°C for 45 min. Enzymes were inactivated as above. Chymotrypsin reactions were buffered using 20 mmol/l Tris-HCl, pH 8.0, while the pepsin reactions were carried out in 0.1 mol/l HCl, pH 3.0. Separate controls containing peptide, pepsin or chymotrypsin were treated in the same manner as the test sample. The E:S ratio was 1:60. Acid-washed glassware was used throughout.

2.4. Fractionation of hydrolysate peptides by solid-phase extraction

One milliliter of the tryptic digest of the β-lg enriched substrate (80 mg/ml protein) was applied to the C_{18} Bond Elut column. Bond Elut® C_{18} solid-phase extraction columns (2.8 ml) were from Varian Sample Preparation Products (Harbour City, CA). Peptide material was slowly eluted from this column by stepwise elution (2.8 ml) with increasing concentrations of acetonitrile (ACN) in trifluoroacetic acid (TFA, 0.1% (v/v)).

2.5. Reversed-phase high performance liquid chromatography of solid-phase extraction fractions

Reversed-phase (RP) HPLC was performed on a Phenomenex (Phenomenex Ltd., Macclesfield, Chesire, UK) C_{18} column (250×3.2 mm, 5 μm), equilibrated with solvent A (0.1% TFA in H_2O) and elution was with a linear gradient to 80% solvent B (60% acetonitrile, 40% H_2O, 0.1% TFA) during the first 50 min, to 90% in the next 10 min, to 100% in the next 5 min and the column was re-equilibrated at 100% solvent A for the last 20 min. Runs were conducted at room temperature using a Shimazu® HPLC system (Shimadzu Corp., Analytical Instruments, Nakagyo-ku, Kyoto, Japan), the flowrate was 0.30 ml min^{-1}. The injection volume was 5 μl.

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2.6. Characterisation of ACE inhibition

ACE inhibitory activity was measured spectrophotometrically using hippuryl-L-histidyl-L-leucine as the substrate for ACE [9]. Initial screening of β-lg hydrolysates was carried out using a single assay to give an index of ACE inhibition. In general, 50 μl of a 10 mg/ml solution of hydrolysate was taken to screen for ACE inhibitory activity. The IC_{50} value was expressed in terms of milligrams of freeze-dried hydrolysate per litre. The % inhibition versus Log_{10} [hydrolysate] (mg/l) curves or the % inhibition versus Log_{10} [peptide] (μmol/l) curves, in the case of synthesised peptide, were constructed using at least seven separate analyses.

3. Results and discussion

The tryptic hydrolysate of the enriched fraction of β-lg, which had a DH value of 6.52%, gave an ACE inhibition index of 84.3%. Unhydrolysed β-lg gave an ACE inhibition index value less than 10% (Table 1). Hydrolysis is therefore necessary in order to release ACE inhibitory activity/peptides from within the sequence of β-lg. The peptide material eluting between 20 and 25% ACN from the solid-phase extraction of the tryptic hydrolysate of β-lg, having an ACE inhibition index of 93.6% and an IC_{50} value of 159.8 mg/l, was analysed using mass spectroscopy. From the mass spectroscopy profile in Fig. 1A it is seen that this fraction mainly contains a peptide giving a charge to mass ratio of 838.4. This approximates very well with the theoretical mass i.e. 837.05 of f(142–148) corresponding to peptide 17 formed during the tryptic digestion of bovine β-lg. This peptide, f(142–148), was synthesised and shown to have a charge/mass ratio of 836.4 (Fig. 1B). This synthetic peptide had an IC_{50} value of 42.6 μmol/l (Table 1). To our knowledge, this is the most potent β-lg-derived ACE inhibitory peptide reported to date. Tyr–Leu, a peptide derivative of β-lactorphin (β-lg f(102–105)) had an IC_{50} of 122.1 μmol/l [6]. The IC_{50} value obtained for Captopril (D-3-mercapto-2-methylpropyl-L-proline) was in accordance with that reported previously (Table 1) [10].

Much speculation has taken place 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 [11,12]. Furthermore, structure-activity data suggests that a positive charge, as on the guanidino group of the C-terminal Arg, contributes substantially to the ACE inhibitory potency of several peptides [13]. The sequence of β-lg f(142–148) appears to reflect these observations. However, the C-terminal tripeptide His–Ile–Arg f(146–148) gave an IC_{50} value of 953.5 μmol/l and an IC_{50} value of 695.5 μmol/l was reported for the related Ile–Arg dipeptide [6]. In common with several food protein-derived ACE inhibitory peptides, β-lg f(142–148) contains a proline residue. Proline residues were

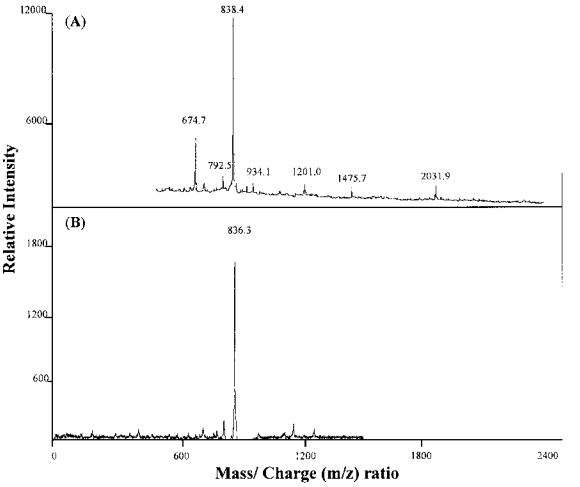

Fig. 1. Mass spectroscopy profiles of (A) peptide material eluting between 20 and 25% acetonitrile following C_{18} solid-phase extraction of a tryptic digest of β-lactoglobulin and (B) of synthetic peptide corresponding to β-lactoglobulin f(142–148).

suggested to contribute to the potency of ACE-inhibitory peptides from food protein sources [12,14,15]. It may well be that the C-terminal (arginine residue) in addition to an internal proline residue contribute significantly to the potency of ACE inhibitory peptides. It was not possible to obtain the Ala–Leu–Pro–Met tetrapeptide using Fmoc synthesis.

In order for peptides to exert a physiological effect, they must interact with ACE at various sites around the body. One of the first requirements for this physiological effect is that inhibitory peptides should be resistant to further attack by digestive proteinases. The synthetic β-lg f(142–148), shown to be present in a tryptic digest of β-lg, was incubated in the presence of pepsin and chymotrypsin in order to investigate the possible formation of further hydrolysis products. The RP-HPLC profiles obtained following incubation of β-lg f(142–148) with pepsin and chymotrypsin are given in Fig. 2.

It appeared from RP-HPLC analysis that no hydrolysis of f(142–148) occurred following incubation with pepsin (Fig. 2a,d,e). RP-HPLC profiles indicate that a low level of hydrolysis occurred following incubation of f(142–148) with chymotrypsin (Fig. 2a,b,c). The low degree of hydrolysis was indicated by two 'extra' peaks in the peptide profile (Fig. 2c). However, the intensity of the peaks corresponding to the synthetic peptide changed only slightly, indicating a low degree of chymotryptic hydrolysis of f(142–148). The tryptic peptide Ala–Leu–Pro–Met–His–Ile–Arg has sites which are poten-

Table 1
ACE inhibitory activity of β-lactoglobulin-derived tryptic hydrolysate/peptide

Sample/peptide sequence	ACE Inhibition Index (%)	IC_{50}[a] (μmol/l)
Unhydrolysed β-lg	9.6	–
Unfractionated β-lg hydrolysate	84.3	–
20–25% ACN peptide elution	93.6	159.8[b]
Ala–Leu–Pro–Met–His–Ile–Arg[c]	–	42.6
Captopril	–	0.006

[a] IC_{50} is defined as that amount of peptide which causes 50% inhibition of ACE using hippuryl-histidyl-leucine as substrate.
[b] Expressed in mg/l.
–, Not determined.
[c] Corresponding to synthetic β-lg f(142–148).

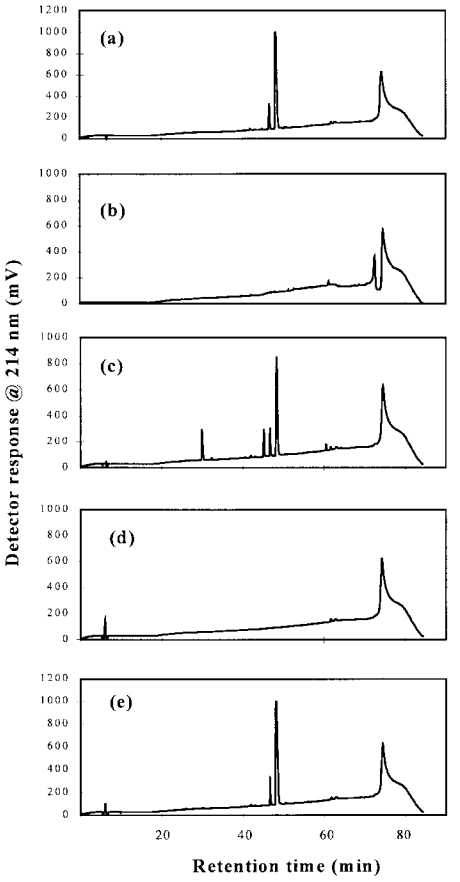

Fig. 2. RP-HPLC (C_{18}) profiles detected at 214 nm of (a) peptide f(142–148) of β-lactoglobulin, (b) chymotrypsin control, (c) peptide incubated with chymotrypsin, (d) pepsin control, and (e) peptide incubated with pepsin. Acetonitrile gradient conditions are detailed in Section 2.

tially susceptible to hydrolysis by chymotrypsin. However, it would be expected that the peptide is too short (i.e. 7 amino acid residues) for further hydrolytic action by endoproteinases. Furthermore, reaction conditions with the high E:S ratio of 1:60 may have forced the limited hydrolysis of f(142–148) by chymotrypsin. It is known that food-derived bioactive peptides are often resistant to further degradation by gastric or pancreatic proteinases.

The whey protein-derived hydrolysate/peptide reported here does not have the potency of Captopril (IC_{50} = 0.006 μmol/l), a drug commonly used in the treatment of hypertension [10]. However, the IC_{50} values found for the whey protein hydrolysate/peptide in this study are comparable to those found for casein hydrolysates/peptides shown to have an in vivo antihypertensive effect following oral ingestion by rats [16]. Furthermore, following a study on the oral administration of a tryptic casein hydrolysate to normotensive and hypertensive human volunteers it has been reported that food-derived peptides having IC_{50} values within the 100–500 μmol/l range had potential significant physiological effects as antihypertensive agents [17]. It is possible therefore that the β-lg hydrolysate/peptide reported herein could find application as nutraceuticals in the prevention of hypertension. A nutraceutical has been defined as a food or part of a food which provides medical or health benefits, including the prevention and treatment of disease [18]. There is an obvious need to perform clinical studies with human volunteers on the potential antihypertensive effect of whey protein-derived hydrolysates/peptides before considering the exploitation of these peptides in physiologically functional foods.

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