The Impact of Fermentation and In Vitro Digestion on the Formation of Angiotensin-I-Converting Enzyme Inhibitory Activity from Pea and Whey Protein

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ABSTRACT

Pea and whey protein were fermented by *Lactobacillus helveticus* and *Saccharomyces cerevisiae* in monoculture and in combination at 28 and 37°C in order to release angiotensin-I-converting enzyme (ACE) inhibitory peptides. The fermentation products were subjected to in vitro gastrointestinal digestion, and the digests of nonfermented samples served as controls. After fermentation, the ACE inhibitory activity (%) increased by 18 to 30% for all treatments, except for the fermentations of whey protein with *Saccharomyces cerevisiae* at 28°C, where no significant change was observed. After digestion, however, both fermented and nonfermented samples reached maximum ACE inhibitory activity. The whey digests tended to have lower (50%) inhibitory concentrations (IC₅₀; 0.14 to 0.07 mg/ml), hence, higher ACE inhibitory activity, than the pea digests (0.23 to 0.11 mg/ml). The nonfermented whey protein digest showed the highest ACE inhibitory activity of all. For pea protein, the nonfermented sample had the lowest IC₅₀ value. These results suggest that in vitro gastrointestinal digestion was the predominant factor controlling the formation of ACE inhibitory activity, hence, indicating its importance in the bioavailability of ACE inhibitory peptides.

(**Key words:** ACE inhibitory peptide, fermentation, gastrointestinal digestion)

Abbreviation key: ACE = angiotensin-I-converting enzyme, GRAS = generally recognized as safe, IC₅₀ = 50% inhibitory concentration, MWCO = molecular weight cut off, SHR = spontaneously hypertensive rats.

INTRODUCTION

Hypertension is a major risk factor for the development of cardiovascular diseases, which is one of the

main causes of mortality in Western countries (Duprez et al., 2002). Diet and lifestyle modification represent effective tools in the prevention of hypertension. In the treatment of the disease, these diet and lifestyle changes can decrease requirements of antihypertensive medication, as well as have beneficial effects related to hypertension not remedied by most drugs (Hermansen, 2000). In this respect, functional foods with blood pressure-lowering properties have recently received considerable attention.

Angiotensin-I-converting enzyme (ACE; EC 3.4.15.1) is a dipeptidyl carboxypeptidase that elevates blood pressure by producing the vasoconstrictor angiotensin II and degrading the vasodilator bradykinin (Campbell, 1987). ACE inhibitory peptides have already been isolated from many food proteins (Dziuba et al., 1999; Fitzgerald and Meisel, 2000). Fermentation and/or digestion are popular food processing steps to release these bioactive or functional peptides from food proteins (Abubakar et al., 1998; Gobetti et al., 2000). After oral administration, the ACE inhibitory peptides may exert an antihypertensive effect, provided they pass the gastrointestinal digestive and absorptive system and reach the cardiovascular system in an active form.

Recently, certain functional foods containing ACE inhibitory peptides have been shown to act as an additional or alternative treatment in hypertension. Daily administration of Calpis sour milk to hypertensive human subjects significantly reduces their blood pressure. The antihypertensive effect of this milk fermented with a starter containing *Lactobacillus helveticus* and *Saccharomyces cerevisiae*, is due to the presence of the ACE inhibitory peptides Val-Pro-Pro and Ile-Pro-Pro, contained in the primary structure of β -casein, and β -casein and κ -casein, respectively (Takano, 1998). Moreover, long-term intake of Val-Pro-Pro and Ile-Pro-Pro, or a sour milk containing these tripeptides attenuates the development of hypertension in spontaneously hypertensive rats (SHR), suggesting a preventive role of ACE inhibitory peptides in hypertension as well (Sipola et al., 2001).

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Pea protein is a valuable protein for human nutrition with the advantage of a good balanced profile of amino acids, a high content in the essential amino acid lysine (82 g/kg protein) and good solubility. In addition, it is an environmentally friendly, low-input crop and may represent an alternative to soy. ACE inhibitory peptides derived from pea protein have not yet been reported in literature. In a previous study, high ACE inhibitory activity in a tryptic digest of pea protein isolate was found, suggesting that the pea may be an alternative source of ACE inhibitory peptides (Vermeirssen et al., 2002b). Fermentation was investigated as a means to produce ACE inhibitory peptides, and the ACE inhibitory activity derived from pea protein was compared to the one of whey protein, a known source of ACE inhibitory peptides (Pihlanto-Leppälä, 2001). Eight lactobacilli and *Saccharomyces cerevisiae*, microorganisms that are already used in food processing were used to inoculate the protein media. The combination of yeast and lactic acid bacteria is often used in fermented milk-based products as several synergistic interactions may occur (Viljoen, 2001). The importance of the gastrointestinal proteases pepsin (A, EC 3.4.23.1), trypsin (EC 3.4.21.4), and α -chymotrypsin (EC 3.4.21.1) on the formation and/or degradation of ACE inhibitory peptides was also assessed.

MATERIALS AND METHODS

Products

The pea protein isolate Pisane HD and the (rennet) whey protein isolate Lacprodan DI-9213 were obtained from Cosucra SA (Fontenoy, Belgium) and Acacris Belgium NV (Londerzeel, Belgium), respectively. Thiamin, pyridoxine, D-pantothenic acid, inositol, D-biotin, nicotinic acid, riboflavin, pepsin (P 6887), trypsin (T 1426), α -chymotrypsin (C 4129), trichloroacetic acid solution (490-10), ACE reagent (305-10), ACE control-E (A 7040), and 1 kg/L trifluoroacetic acid solution (30,203-1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nonspecified products were analytical grade and came from VWR International (Zaventem, Belgium).

Fermentation

The following lactobacilli were used in the screening fermentations: *Lactobacillus fermentum* LMG8900, *Lactobacillus gasserii* LMG9203, *Lactobacillus oris* LMG9848, *Lactobacillus reuteri* LMG9213, *Lactobacillus acidophilus* LMG7943, *Lactobacillus plantarum* LMG9211, *Lactobacillus plantarum* LMG9212, and *Lactobacillus helveticus* LMG 11474 (LMG Culture Collection, Ghent University, Belgium). The first seven microorganisms originated from humans, while the last

was derived from a Swiss cheese starter. In the subsequent fermentations, *Saccharomyces cerevisiae* (commercial baking yeast) was used as well. Lactobacilli were propagated in MRS broth (de Man, Rogosa, Sharpe; Oxoid, Basingstoke, UK) under micro-aerophilic conditions at 37°C for 24 h and baking yeast in YPD broth (10 g/L Yeast extract; Oxoid), 10 g/L Peptone bacteriological (Oxoid; 20 g/L D-glucose) under aerobic conditions at 28°C for 24 h.

The screening fermentations were carried out with pea protein only, for 48 h at 37°C. Fermentations with *Lactobacillus helveticus* and *Saccharomyces cerevisiae* occurred in monoculture at their optimal temperature (37 and 28°C, respectively) and in combination (37 and 28°C) for 48 h on both pea and whey protein. At the start of the fermentation, the selected microorganisms were added to the fermentation medium under sterile conditions in a concentration of 6.3 log₁₀ cfu/ml for *L. helveticus* and 5.7 log₁₀ cfu/ml for *S. cerevisiae*. Samples at the start of the fermentation were taken for analysis. Then the inoculated fermentation medium was incubated at 28 or 37°C for 48 h, after which fermented samples were taken. Finally, the fermented medium was subjected to an in vitro gastrointestinal digestion and a last sampling took place. Fermentation experiments with *L. helveticus* or/and *S. cerevisiae* were repeated at least three times.

The fermentation medium was composed as follows: 40 g/L protein, 20 g/L D-glucose, 10 mL/L vitamin solution, 10 mL/L salt solution, and 0.1 M sodium phosphate buffer, pH 6 to 6.5. To avoid the Maillard reaction, the protein solution and the rest solution, consisting of D-glucose and salt solution in sodium phosphate buffer, were autoclaved separately for 15 min at 121°C, 101.3 kPa overpressure. The salt solution contained (per liter) 5 g of MgSO₄·7H₂O, 5 g of KH₂PO₄, 1 g of CaCl₂·2H₂O, and 0.5 g of MnSO₄·H₂O. The vitamin solution consisted of (per liter) 40 mg of thiamine, 40 mg of pyridoxine, 40 mg of D-pantothenic acid, 200 mg of inositol, 2 mg of D-biotin, 40 mg of nicotinic acid, and 40 mg of riboflavin. It was added to the autoclaved fermentation medium after sterilization by 0.22 μ m filtration (Millipore, Bedford, MA). Before inoculation, the pH of the 100 ml whey protein fermentation medium was adjusted to pH 6 to 6.5 by the addition of 450 μ l 10 M NaOH.

Digestion

The conditions of the physiological digestion were based on literature (Gauthier et al., 1986; Ganong, 1997; De Boever et al., 2000). To simulate the digestion in the stomach, the pH of the fermented medium was adjusted to 2 with 10 M and 1 M HCl under rigorous mixing. Subsequently, pepsin was supplemented in an

enzyme to substrate ratio of 1 over 250, and the medium was incubated on a shaker for 2 h at 37°C. Next, the small intestinal digestion was simulated by setting the pH at 6.5 with 10 M and 1 M NaOH under rigorous mixing and by addition of trypsin and α -chymotrypsin, both in an enzyme to substrate ratio of 1 over 250, followed by another incubation for 2.5 h on a shaker at 37°C. At the end of digestion, the pH was adjusted to 5 with 10 M and 1 M HCl. As this is a pH near the isoelectrical point for both proteins (pea: pH 4.5, whey: pH 4 to 5), a standardized and clear separation was obtained by subsequent centrifugation.

Followup of the Fermentation

At the start and the end of the fermentation, pH was measured using a 744 pH Meter (Metrohm, Herisau, Switzerland), and plate counts were performed after 72 h of incubation at 37°C on Rogosa agar (Oxoid) and at 28°C on YPD agar (YPD broth + 20 g/L agar) for lactobacilli and *S. cerevisiae*, respectively.

Degree of Proteolysis

The degree of proteolysis was determined by the ratio of the nonprotein Kjeldahl nitrogen to the total Kjeldahl nitrogen. Samples for nonprotein nitrogen determination were treated with trichloroacetic acid solution to a final concentration of 60 g/L, shaken for 5 min, and then centrifuged at $12,000 \times g$ for 10 min at 4°C. This supernatant and a sample for total nitrogen determination were stored at -70°C prior to analysis.

ACE Inhibitory Activity

Samples for ACE inhibition were centrifuged at $10,000 \times g$ for 15 min at 4°C, the supernatant was frozen in liquid nitrogen, and stored at -70°C. Next, the frozen samples were lyophilized during 3 d to obtain a dry powder. Lyophilized powder (10 mg) was dissolved in 1 ml of demineralized water and analyzed by the ACE inhibition assay as described by Vermeirssen et al. (2002b), with the modification that pure ACE from porcine kidney (ACE control-E), was used. By this spectrophotometric method, ACE inhibition is measured using the substrate furanacryloyl-Phe-Gly-Gly. The reaction mixture contained 500 μ l ACE reagent, 300 μ l demineralized water (blank) or inhibitor solution, and 300 μ l ACE control-E. The decrease in absorbance at 340 nm over 5 min corresponded to an ACE activity (U/L) as determined by the standard curve, and a percentage of ACE (inhibitory) activity, assuming that the ACE (inhibitory) activity of the blank is 100% (0%). The detection limit of the ACE inhibitory activity assay is 11%.

When ACE inhibitory activity exceeded 80%, dilution series were made to determine the 50% inhibitory concentration (IC_{50}) value. The latter is defined as the concentration of inhibitory compound in the assay that inhibits 50% of the ACE activity. Dose-activity curves were generated for doses of inhibitor versus ACE (inhibitory) activity. The IC_{50} value was obtained by fitting the data to a four parametric logistic model using the Marquardt-Levenberg algorithm (Sigmaplot 4.0, SPSS Inc., Chicago, IL).

$$y = \min + \frac{\max - \min}{1 + 10^{[\log IC_{50} - x] \cdot \text{hill slope}}}$$

In this equation, y represents the ACE inhibitory activity (%); x, the logarithm of the concentration of inhibitor (mg/ml); min, the baseline of 0% inhibition; max, the plateau of 100% inhibition; and hill, slope the slope of the curve at the transition center IC_{50} . ACE inhibition analysis was repeated three times per sample. Captopril was used to validate the assay and showed a 50% inhibitory concentration (IC_{50}) of 7.5 nM with a 95% confidence interval of 6.4 to 8.8 nM.

Protease Activity

The protease activity was determined on the supernatant and the redissolved precipitate of the 24-h propagated culture of *L. helveticus* and *S. cerevisiae*, respectively, after centrifugation at $5000 \times g$ for 10 min (n = 3). Activity was assessed by means of a commercially available Universal Protease Substrate spectrophotometric assay (Roche Diagnostics, Basel, Switzerland).

HPLC Analysis

Samples for the HPLC analysis were treated in the same manner as for the ACE inhibition assay. Twenty-milligram lyophilized powder was dissolved in 2-ml milli-Q water (Millipore) and ultrafiltrated-centrifuged in Centricon YM-3000 tubes (molecular weight cut-off (MWCO): 3000 Da; Millipore) for 2 h at $7500 \times g$. The permeate was analyzed by reversed-phase HPLC on a Prosphere 300 Å C_{18} column (250 $\times$ 4.6 mm, 5 μ m; Alltech Associates, Deerfield, IL) and a Dionex (Sunnyvale, CA) HPLC with an autosampler ASI-100, pump series P580, STH585 column oven, UV-VIS detector UVD340S operating at 210 nm, and Chromeleon 6.0 software. Elution was at 25°C with a flow rate of 1 ml/min, a linear gradient from 90% solvent A (H_2O + 1 g/L TFA) to 50% solvent B (acetonitrile + 0.85 g/L TFA) in 30 min, again to 90% solvent A for the next 20 min, and remaining at 90% solvent A for the last 10 min.

Sonication

Sonication (Sonicator 250, Branson, G. Heinemann, Schwäbisch Gmünd, Germany) was performed in the ice-cooled, 24-h propagated culture of *L. helveticus*, *S. cerevisiae*, or a combination of both [1/1 (vol/vol) propagated culture of both microorganisms] to release the intracellular proteases from the lysed cells in the medium. Operational parameters resulting in the highest protease activity as obtained with the commercial protease assay were found to be: time = 12 min, output control = 3 and duty cycle = 70%. A 40-g/L whey or pea protein solution in demineralized water was incubated with 10-ml/L lysed cell suspensions of *S. cerevisiae* at 28°C or *L. helveticus* or both at 37°C for 24 h.

Statistical Analysis

All values, except IC_{50} , are reported as mean $\pm$ standard error of the mean ($n_{\min} = 3$). Some missing values (2% of the dataset) were replaced by the average. A paired Student's *t*-test studied the significance of changes in pH, lactobacilli, and yeast counts during fermentation. To exclude the initial effect of the protein itself, the statistical analysis was performed on the changes in degree of proteolysis and ACE inhibitory activity during fermentation, and fermentation and digestion. By means of the general linear model procedure (Minitab 11.21, State College, PA) significant differences in type of protein and type of fermentation were assigned. When there was a significant type of protein $\times$ type of fermentation interaction, for both proteins a Oneway ANOVA analysis was carried out in type of fermentation. All data used in the variance analysis met the homogeneity of variance requirement assessed by Bartlett's test for normal and Levene's test for non-normal distributions. Normality was tested by means of the Anderson-Darling criterion. Indication of subgroups was done by Tukey post hoc test ($P < 0.05$). For the comparison of the heteroscedastic log IC_{50} values, obtained from the 4 parametric logistic model, the Games-Howell method was used ($P < 0.05$; Sokal and Rohlf, 1995). IC_{50} values are reported as mean and 95% confidence interval.

RESULTS

Screening of Lactobacilli

Eight different generally recognized as safe (GRAS) lactobacilli were screened for the production of ACE inhibitory activity during fermentation and subsequent in vitro gastrointestinal digestion of pea protein (Table

1). Fermentation with *L. helveticus* yielded the highest ACE inhibitory activity and was, therefore, selected for subsequent experimentation. After in vitro digestion, all ferments reached high to maximal ACE inhibitory activity.

Fermentation by *Lactobacillus helveticus* and/or *Saccharomyces cerevisiae*

Autoclaved pea and whey protein medium were fermented with *L. helveticus* at 37°C, *S. cerevisiae* at 28°C, or a combination of both microorganisms at 28 or 37°C in order to produce ACE inhibitory active ferments. Fermentation was always followed by an in vitro gastrointestinal digestion with pepsin at pH 2 and a combination of trypsin and chymotrypsin at pH 6.5. Nonfermented autoclaved pea and whey protein medium, subjected to in vitro gastrointestinal digestion, served as control experiments.

The presence of *L. helveticus* at its optimal temperature of 37°C initiated a large pH decrease during fermentation (Table 2). The pH after fermentation was lower for pea than for whey ($P < 0.001$). *L. helveticus* counts increased during fermentation in monoculture on both protein media. However, this lactic acid bacterium hardly grew in the presence of the yeast, both at 37 and 28°C, for pea and whey protein (Table 2). *S. cerevisiae* counts increased during fermentation at 28°C, whether or not in the presence of *L. helveticus*. A higher, nonoptimal temperature, e.g., 37°C, was disadvantageous for the yeast. Hence, the growth of both microorganisms was affected by the type of fermentation ($P < 0.001$) but in a different manner. The counts of both microorganisms did not differ significantly between the two proteins.

Degree of Proteolysis and ACE Inhibitory Activity in Ferments and Digests

At the start of the fermentation process, the degree of proteolysis for pea, $6.7 \pm 0.4\%$, was lower than for whey, $17.9 \pm 0.5\%$ ($P < 0.001$). The ACE inhibitory activity of the fermentation medium of pea amounted to $16 \pm 2\%$, while for whey this was $63 \pm 2\%$ ($P < 0.001$). Figure 1 shows the degree of proteolysis and Figure 2 the ACE inhibitory activity, after fermentation and after fermentation and digestion for both proteins. The statistical analysis, however, was performed on the changes of these parameters during fermentation, and fermentation and digestion, in order to exclude the initial degree of proteolysis and ACE inhibitory activity of the protein itself.

The change in degree of proteolysis during fermentation was significantly different between the two pro-

Table 1. Angiotensin-I-converting enzyme (ACE) inhibitory activity (%) after fermentation of pea protein medium by different lactobacilli at 37°C and subsequent in vitro digestion. The data represent the result of a single experiment.

Strain	After fermentation ^{1,2}	After in vitro digestion ^{1,3}
<i>Lactobacillus fermentum</i> LMG8900	12	90
<i>Lactobacillus gasseri</i> LMG9203	12	96
<i>Lactobacillus oris</i> LMG9848	16	100
<i>Lactobacillus reuteri</i> LMG9213	9	96
<i>Lactobacillus acidophilus</i> LMG7943	13	93
<i>Lactobacillus plantarum</i> LMG9211	7	83
<i>Lactobacillus plantarum</i> LMG9212	11	99
<i>Lactobacillus helveticus</i> LMG11474	37	96

¹The concentration of ferment or digest in the assay was 2.73 mg/ml.

²The initial ACE inhibitory activity of the pea fermentation medium was 16 ± 2%.

³The ACE inhibitory activity of nonfermented digested pea fermentation medium amounted to 99.3 ± 0.7%.

teins ($P = 0.033$) and between the different types of fermentation ($P < 0.001$). The degree of proteolysis increased during fermentation maximally by 15%, in the monoculture fermentation with *L. helveticus* (Figure 1). The fermentation with both *L. helveticus* and *S. cerevisiae* at 37°C also increased the degree of proteolysis, more in the pea than in the whey protein medium. In the presence of *S. cerevisiae* at 28°C, no significant change could be observed, except for the monoculture fermentation with *S. cerevisiae* on whey protein (W-Y28), where the degree of proteolysis decreased slightly after fermentation ($P < 0.05$). After in vitro digestion, the degree of proteolysis increased sharply in all samples, and the degree of proteolysis in the nonfermented media did not differ from the fermented ones ($P = 0.625$). The less-pronounced increase in degree of proteolysis for the whey protein during digestion compared with

the pea protein ($P < 0.001$), can partially be explained by the higher initial degree of proteolysis for the whey protein fermentation medium.

For the pea protein, the type of fermentation did not influence the increase in ACE inhibitory activity after fermentation ($P = 0.539$; Figure 2). For the whey protein, on the other hand, the presence of *L. helveticus* and a temperature of 37°C resulted in increased ACE inhibitory activity after fermentation, whereas the presence of *S. cerevisiae* and a temperature of 28°C did not ($P < 0.001$). In vitro digestion increased the ACE inhibitory activity significantly, and both nonfermented and fermented samples reached the maximum level of 100% ($P = 0.959$). Somewhat due to the high initial ACE inhibitory activity of the whey protein, the increase during digestion was smaller than with the pea protein ($P < 0.001$).

Table 2. pH and plate counts ($\log_{10}$ cfu/ml) of *Lactobacillus helveticus* and *Saccharomyces cerevisiae* after 48 h fermentation for the different types of fermentations (LH37: *L. helveticus* at 37°C, Y28: *S. cerevisiae* at 28°C, LHY37: *L. helveticus* and *S. cerevisiae* at 37°C, LHY28: *L. helveticus* and *S. cerevisiae* at 28°C), for both pea and whey protein ($n_{\min} = 3$).

		pH ¹	<i>L. helveticus</i> ²	<i>S. cerevisiae</i> ³
Pea	LH37	3.5 ± 0.1***a	7.8 ± 0.4***a	/
	Y28	5.5 ± 0.2 ^b	/	7.1 ± 0.2***a
	LHY37	3.6 ± 0.1***a	6.5 ± 0.3 ^b	5.6 ± 0.5 ^b
	LHY28	5.4 ± 0.3 ^b	6.6 ± 0.4 ^b	7.7 ± 0.2***a
Whey	LH37	3.9 ± 0.1***a	7.4 ± 0.3***a	/
	Y28	6.1 ± 0.0***b	/	7.6 ± 0.1***a
	LHY37	4.3 ± 0.2***a	6.3 ± 0.3 ^b	5.1 ± 0.2***b
	LHY28	5.9 ± 0.1* ^b	6.3 ± 0.2 ^b	7.6 ± 0.3***a

¹Initial pH of the fermentation medium was 6.1 ± 0.0 for pea and 6.3 ± 0.0 for whey.

²*L. helveticus* counts at the start of the fermentation were 6.3 ± 0.1 $\log_{10}$ cfu/ml, for both proteins.

³*S. cerevisiae* counts at the start of the fermentation were 5.7 ± 0.1 $\log_{10}$ cfu/ml, for both proteins.

/ = not applicable.

* = significant from the start value: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

a, b = division in subsets by the factor "type of fermentation" by means of the Tukey post hoc test ($P < 0.05$).

Figure 1. Degree of proteolysis (%) after fermentation, and after fermentation and digestion of pea (P) and whey (W) protein. (LH37: *Lactobacillus helveticus* at 37°C, Y28: *Saccharomyces cerevisiae* at 28°C, LHY37: *L. helveticus* and *S. cerevisiae* at 37°C, LHY28: *L. helveticus* and *S. cerevisiae* at 28°C, n-ferm: non-fermented). Black bars = after fermentation, line pattern bars = after fermentation and digestion; straight, dashed lines indicate the initial degree of proteolysis of the pea and whey medium.

^{a, b, c} = division in subsets of the change in degree of proteolysis during fermentation and fermentation and digestion, by the factor "type of fermentation" by means of the Tukey post hoc test ($P < 0.05$).

Figure 2. Angiotensin converting enzyme (ACE) inhibitory activity (%) after fermentation, and after fermentation and digestion of pea (P) and whey (W) protein. (LH37: *Lactobacillus helveticus* at 37°C, Y28: *Saccharomyces cerevisiae* at 28°C, LHY37: *L. helveticus* and *S. cerevisiae* at 37°C, LHY28: *L. helveticus* and *S. cerevisiae* at 28°C, n-ferm: non-fermented). Black bars = after fermentation, line pattern bars = after fermentation and digestion; straight, dashed lines indicate the initial ACE inhibitory activity of the pea and whey medium.

^{a, b, c} = division in subsets of the change in ACE inhibitory activity during fermentation, and fermentation and digestion, by the factor "type of fermentation" by means of the Tukey post hoc test ($P < 0.05$).

Table 3. 50% inhibitory concentrations (IC₅₀) (mg/ml) and 95% confidence interval for the different types of fermentation for pea and whey protein. (LH37: *Lactobacillus helveticus* at 37°C, Y28: *Saccharomyces cerevisiae* at 28°C, LHY37: *L. helveticus* and *S. cerevisiae* at 37°C, LHY28: *L. helveticus* and *S. cerevisiae* at 28°C, n-ferm: non-fermented).

		IC ₅₀	95% confidence interval
Pea	LH37	0.23	0.14 – 0.39
	Y28	0.18	0.13 – 0.25
	LHY37	0.16*	0.15 – 0.18
	LHY28	0.11	0.09 – 0.13
	n-ferm	0.12	0.08 – 0.16
Whey	LH37	0.14	0.12 – 0.16
	Y28	0.08	0.06 – 0.11
	LHY37	0.11	0.09 – 0.13
	LHY28	0.10*	0.08 – 0.12
	n-ferm	0.07	0.04 – 0.10

* = Significantly different from each other ($P < 0.05$) as determined by the Games-Howell method.

The degree of proteolysis did not correlate with the ACE inhibitory activity: although the pea ferments at 28°C showed a lower degree of proteolysis compared with the pea ferments at 37°C, the ACE inhibitory activity of both groups did not differ.

The IC₅₀ value, the concentration of inhibitory compound necessary to inhibit the ACE enzyme for 50%, enabled to further distinguish the ACE inhibitory activity of the digests (Table 3). Due to nonequal and, for some samples, large variances, the combined fermentation with *L. helveticus* and *S. cerevisiae* of the pea protein at 37°C and the combined fermentation with *L. helveticus* and *S. cerevisiae* of the whey protein at 28°C were the only samples that were significantly different. However, the whey digests tended to have a lower IC₅₀, hence a higher ACE inhibitory activity, than the corresponding pea digests. The presence of the yeast at 28°C seemed to decrease the IC₅₀ value. The lowest IC₅₀ value was obtained in the nonfermented whey protein medium. For pea protein, the nonfermented sample had the one of the lowest IC₅₀ value. Hence, the nonfermented samples were at least as ACE inhibitory active as the fermented ones.

Protease Activity

Protease activity in the 24-h propagated culture of *L. helveticus* and *S. cerevisiae* was measured on the supernatant and the cell precipitate in order to identify the origin of protease activity. The protease activity in the supernatant measured 0.80 ± 0.01 U/L for *L. helveticus* and 0.53 ± 0.07 U/L for *S. cerevisiae*. The cell precipitate of *L. helveticus* had a protease activity of 0.39 ± 0.03 U/L, whereas the one of *S. cerevisiae* had an activity of 0.55 ± 0.13 U/L.

HPLC Profiles

The HPLC profile of the samples provided a fingerprint of the small peptides (MWCO ultrafiltration-centrifugation = 3000 Da) present in the digests. No real differences between the chromatograms of the fermented and the nonfermented digests were observed. For both proteins, the HPLC chromatogram of one fermented digest and the nonfermented digested medium are compared in Figure 3. The type of protein and the digestion step determined the HPLC profile and, hence, the formation of small peptides with possible ACE inhibitory effects.

Contribution of Intracellular Proteases

Nonautoclaved 40 g/L pea and whey protein solution had a degree of proteolysis of 2.4 ± 0.4 and $12.9 \pm 0.7\%$ ($P < 0.001$), respectively, and an ACE inhibitory activity of 10.0 ± 0.7 and $15 \pm 2\%$ ($P = 0.083$), respectively. After 24-h incubation with a suspension of lysed cells of *L. helveticus* at 37°C, *S. cerevisiae* at 28°C, and a combination of both at 37°C, the degree of proteolysis increased only for the pea protein and amounted to 30% at the end of digestion for all suspensions. Similarly, for the pea protein only, an increase in ACE inhibitory activity was observed. The ACE inhibitory activity reached after incubation 80% for *S. cerevisiae*, 30% for *L. helveticus*, and 40% for the combination lysed cells. In the whey protein digests, no change in degree of proteolysis, nor ACE inhibitory activity, could be detected.

DISCUSSION

In this study, pea and whey protein were fermented by lactobacilli and yeast in order to release ACE inhibitory peptides that could lower the blood pressure in vivo. Subsequently, the impact of an in vitro gastrointestinal digestion on the ACE inhibitory activity was evaluated.

From the eight GRAS lactobacilli selected, only *L. helveticus* produced considerable ACE inhibitory activity after fermentation of pea protein medium. *L. helveticus*, whether or not in combination with *S. cerevisiae*, is known for its capacity to form ACE inhibitory peptides from milk proteins (Nakamura et al., 1995; Yamamoto et al., 1999). Fermented milk prepared by most strains of *L. helveticus* significantly lowers the blood pressure in SHR upon oral administration, while milk fermented by other species of lactic acid bacteria, among which was a *L. acidophilus* strain, does not display significant antihypertensive effects (Yamamoto et al., 1994b). According to the same study, ACE inhibitory activity in most of the whey fractions of the milk fermented with *L. helveticus* is also higher than in the other fermented

Figure 3. HPLC-profile of the digested nonfermented pea (P-n-ferm) and whey (W-n-ferm) medium, pea medium fermented with *L. helveticus* at 37°C (P-LHY37) and whey medium fermented with *S. cerevisiae* at 28°C (W-LHY28).

milks. Nevertheless, ACE inhibitory peptides have been isolated from different dairy products started by lactic acid bacteria: e.g., *Lactobacillus delbrueckii* subsp. *bulgaricus* SS1, *Lactococcus lactis* subsp. *cremoris* FT4, *Lactobacillus acidophilus*, bifidobacteria, and *Streptococcus thermophilus* (Gobbetti et al., 2000; Saito et al., 2000; Ryhnen et al., 2001).

Based on the results from the screening and literature, *L. helveticus* and *S. cerevisiae* were subsequently used to initiate fermentations in monoculture and in combination on pea and whey protein. The ACE inhibitory activity after fermentation is not a good selection criterion, because the physiological transformations in the human body, which determine the bioavailability of the ACE inhibitory peptides, are not taken into consideration. However, often only the food-processing step is envisaged (Haileselassie et al., 1999; Kim et al., 2001). By incorporation of the in vitro gastrointestinal digestion, the measured ACE inhibitory activity relates more closely to the ACE inhibitory activity in the human body. To exert an antihypertensive effect, the peptides also have to pass the intestinal barrier to arrive in the blood in an active form, an issue which has been the scope of another paper (Vermeirssen et al., 2002a). From this study, it was hard to make a selection after in vitro digestion, since almost all fermented digests reached maximum ACE inhibitory activity.

The lactic acid bacterium *L. helveticus* was metabolically more active at 37°C, and this resulted in more lactic acid production and, hence, a larger pH decrease. Although *L. helveticus* and *S. cerevisiae* have complex nutritional requirements for growth (Walker, 1998; Hebert et al., 2000), both microorganisms were able to grow on the pea and whey protein medium in monoculture at their optimal temperature. The type of protein did not influence their growth, showing that pea and whey protein both could serve as a nitrogen source for *L. helveticus* and *S. cerevisiae*. In the combined fermentations, the yeast did not favor the growth of *L. helveticus*, which could be due to the production of, e.g., organic acids, antibacterial compounds, ethanol, or competition for nutrients. The counts of *S. cerevisiae*, on the other hand, decreased slightly in the presence of *L. helveticus* at 37°C, which can either be attributed to the high acid production or the high temperature.

The increase in degree of proteolysis observed in the ferments with *L. helveticus* was induced by the action of its proteases, possibly supplemented by a slight acid hydrolysis. *L. helveticus* is known to possess a cell-wall-associated protease, capable of forming antihypertensive peptides from casein (Yamamoto et al., 1994a), and several peptidases (Christensen et al., 1999). However, the protease activity assay found a higher activity in the supernatant than in the cell precipitate, indicating that the cell-wall-associated protease has been released from the cell wall or that other secreted proteases played a major role. Also, the peptide-rich propagation medium may inhibit the synthesis of the cell-wall-associated protease in *L. helveticus* (Hebert et al., 2000). The total potential protease activity of *S. cerevisiae* was similar to the one of *L. helveticus*, but this was not

translated into an increase in degree of proteolysis after fermentation. Generally, *S. cerevisiae* has no extracellular proteases or peptidases to cleave oligopeptides or proteins in the medium, only some specialized strains have (Walker, 1998). However, it seems that the strain used in this study did possess some extracellular protease activity. A nonsignificant increase and even decrease in degree of proteolysis by the yeast can be explained by the consumption of peptides and amino acids present in the medium and by an increase in total protein content due to yeast growth. The gastrointestinal proteases sharply increased the degree of proteolysis, which simulated well the conditions during physiological digestion. The specificity and purity of pepsin, trypsin, and α -chymotrypsin led to the formation of numerous peptides and amino acids. The pea protein was more susceptible to hydrolysis than the whey protein.

Autoclaving had a major impact on the ACE inhibitory activity of the heat labile whey protein. This may be caused by the presence of natural proteases in the whey protein isolate, which are initially activated by the temperature increase during autoclaving. In vitro gastrointestinal digestion released significant ACE inhibitory activity from pea and whey protein medium and ferments. However, despite the growth of the microorganisms, the increase in degree of proteolysis and the increase in ACE inhibitory activity after fermentation, the fermented samples did not show higher ACE inhibitory activity after in vitro digestion than the nonfermented samples. Fermentations ineffective in producing ACE inhibitory activity have been reported in literature. Pihlanto-Leppä et al. (1998) observed ACE inhibitory activity in whey and casein fermented with different lactic acid starters only after digestion by trypsin and pepsin. They attribute their results to the low proteolytic activity of the starters or the specificity of the enzymes in lactic acid bacteria. The formation of bioactive peptides by lactic acid bacteria seems to be a rare event and is recently being debated (Meisel and Bockelmann, 1999). The IC₅₀ values not only confirmed this observation, but even pointed out that fermentation may sometimes adversely affect the ACE inhibitory activity as the nonfermented whey protein digest showed the highest ACE inhibitory activity. The tendency towards higher IC₅₀ values in the fermented digests can be enlightened by the hypothesis that the microbial enzymes split within the sequence of bioactive peptides in the food protein, thereby preventing the gastrointestinal proteases to release them. Therefore, there exists an optimal degree of hydrolysis, above which more ACE inhibitory peptides are degraded than new peptides are formed, decreasing the overall ACE inhibitory activity (Mullally et al., 1997). Hence, no direct relationship between the degree of proteolysis

and the ACE inhibitory activity is possible, especially in the later stages of hydrolysis. However, after fermentation of the whey but not the pea protein, some correlation between the degree of proteolysis and the ACE inhibitory activity was observed. The IC_{50} values of the digests ranged from 0.07 to 0.23 mg/ml, which are slightly more active than the values reported for whey digests by gastrointestinal proteases of 0.35 to 1.73 mg/ml (Pihlanto-Leppä et al., 2000). Differences in assay conditions make it somehow difficult to compare these values. The tendency towards lower IC_{50} values for the whey compared to the pea digests was due to the intrinsic amino acid sequence of the protein.

Furthermore, the HPLC chromatograms of the peptide fraction with MWCO <3000 Da, did not differ between fermented and nonfermented digests. In vitro digestion resulted in the formation of small peptides, which possibly possess ACE inhibitory and antihypertensive effects. Matar et al. (1996), however, concluded that there is a major impact of milk fermentation by *L. helveticus* on subsequent gastrointestinal digestion. The HPLC elution profiles of that study show that fermentation may contribute to the formation of new peptides during in vitro digestion, provided that the pH is controlled at 6.0 during the fermentation, which favors the proteolytic activity of *L. helveticus*. Nevertheless, in other fermentation studies with *L. helveticus* and other lactic acid bacteria that were effective in producing ACE inhibitory peptides, pH was never controlled (Nakamura et al., 1995; Yamamoto et al., 1999; Gobetti et al., 2000).

In the case of the pea protein, the preliminary study on the production of ACE inhibitory activity from lysed cells pointed to the importance of the intracellular proteases from *S. cerevisiae*. Lysis of the cells made these proteases readily available for splicing off the bioactive peptide sequences from the food proteins. Intracellular proteases and peptidases of both *L. helveticus* and *S. cerevisiae* will most likely contribute to degradation of proteins after cell lysis (Meisel and Bockelmann, 1999). High ACE inhibitory activity is identified in skimmed milk digested with cell-free extract of *S. cerevisiae*, and the hydrolysate obtained with the purified protease B shows an IC_{50} of 0.42 mg protein/ml (Roy et al., 2000). An extract of autologous *Lactobacillus casei* cell lysate shows an antihypertensive effect in hypertensive patients, but this is due to the presence of polysaccharide-glycopeptide complexes (Nakajima et al., 1995).

CONCLUSIONS

We were able to produce ACE inhibitory active digests from pea and whey protein. Nevertheless, a fermentation step by *L. helveticus* or/and *S. cerevisiae* did

not improve the release of ACE inhibitory activity after in vitro gastrointestinal digestion. The formation and degradation of ACE inhibitory activity during gastrointestinal digestion and absorption merit further work.

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