**Dairy *Debaryomyces hansenii* strains produce the antihypertensive
casein-derived peptides LHLPLP and HLPLP**

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Abstract

The ability of dairy *Debaryomyces hansenii*, *Kluyveromyces lactis* and *Kluyveromyces marxianus* strains to release the casein-derived antihypertensive sequences RYLGY, AYFYPEL, LHLPLP, HLPLP, VPP and/or IPP was examined. Yeast strains were grown in medium with casein as sole nitrogen source and the yeast casein hydrolysates (CSHs) were analysed by HPLC-MS/MS to search for the six antihypertensive sequences. Only LHLPLP and HLPLP were identified in CSHs and exclusively in *D. hansenii* Dh1 and Dh14 hydrolysates in which both antihypertensive sequences represented approximately 6 (CSH Dh1) and 10% (CSH Dh14) of total peptide content. In addition, a complete analysis of selected CSHs by HPLC-MS/MS allowed the identification of 35 (Dh1) and 46 (Dh14) additional peptides, which could also contribute to the observed *in vitro* ACE inhibitory potency of both hydrolysates (Dh1, $IC_{50} = 13.6 \pm 1.8 \mu\text{g/mL}$; Dh14, $IC_{50} = 17.5 \pm 2.1 \mu\text{g/mL}$) and might confer them multifunctional properties. Finally casein zymography revealed the presence of at least one extracellular protease with a molecular mass of about 50 kDa. In conclusion, the present study contributes to a better insight into bioactive compounds produced by dairy yeasts and shows the feasibility of *D. hansenii* strains to produce antihypertensive casein-derived peptides.

Keywords: Dairy yeasts, *Debaryomyces hansenii*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, casein-derived antihypertensive peptides.

43 **Abbreviations**

44 ACE: angiotensin I-converting enzyme; CSH: casein hydrolysate; GRAS:
45 generally recognized as safe; LAB: lactic acid bacteria; RP-HPLC-MS/MS:
46 reversed phase-high performance liquid chromatography tandem mass
47 spectrometry; RP-HPLC-UV-MS: reversed-phase high performance liquid
48 chromatography-UV and mass spectrometry; SHR: spontaneously hypertensive
49 rat.

50

1. Introduction

Casein is an excellent substrate to produce peptides with angiotensin I converting enzyme (ACE)-inhibitory and antihypertensive effects (Otte, Shalaby, Zakora, Pripp, & El-Shabrawy 2007; Martínez-Maqueda, Miralles, Recio, & Hernández-Ledesma, 2012). Basically, processing of milk proteins with food grade proteolytic preparations or fermentation of milk with lactic acid bacteria (LAB) proteolytic starters have been employed to release antihypertensive peptides. Both approaches have conducted to the development of commercial products based on milk proteins with antihypertensive effects in humans. In fact, one of the most popular functional foods contain the casein-derived ACE-inhibitory tripeptides VPP and IPP, which can be obtained by means of either milk fermentation (Nakamura, Yamamoto, Sakai & Takano, 1995) or enzymatic hydrolysis using microbial proteases (Mizuno, Nishimura, Matsuura, Gotou & Yamamoto, 2004).

We have previously identified casein-derived sequences with antihypertensive activity using both methods. Two novel peptides of sequences RYLGY and AYFYPEL were identified from a peptic casein hydrolysate (Contreras, Carrón, Montero, Ramos, & Recio, 2009.). Both exerted *in vitro* inhibitory effects on ACE activity (IC₅₀ values of 0.71 and 6.58 µM, respectively) and effectively decreased systolic blood pressure after oral administration to spontaneously hypertensive rats (SHRs) at 5 mg/kg of body weight. With respect to the fermentation strategy, the antihypertensive sequence LHLPLP was identified as one of the major peptides responsible for the ACE-inhibitory and antihypertensive effects of fermented milk produced by *Enterococcus faecalis* (Muguerza et al., 2006; Quirós et al., 2007). This sequence showed an

IC₅₀ value of 5.5 ± 0.4 µM against ACE activity and exhibited potent antihypertensive effect at a dose of 2 mg/kg (Quirós et al., 2007). Further characterization suggested the pentapeptide HLPLP as the minimum active form responsible for the antihypertensive effect of LHLPLP (Quirós, Dávalos, Lasunción, Ramos, & Recio, 2008.). Since the pathogenic potential of some *Enterococcus* strains (Franz, Holzapfel, & Stiles, 1999) may hamper their use in the food industry, production of HLPLP by enzymatic hydrolysis with food-grade enzymes was also optimized (Quirós, Hernández-Ledesma, Ramos, Martín-Álvarez, & Recio, 2012).

The impact of proteolytic yeast strains on the release of ACE-inhibitory peptides in fermented milk has been recently described (Chaves-López, Tofalo, Serio, Paparella, Sacchetti, & Suzzi, G, 2012; Chaves-López et al., 2014). We have shown the ability of some proteolytic dairy yeast strains belonging to *Debaryomyces hansenii*, *Kluyveromyces lactis* and *Kluyveromyces marxianus* species to generate casein-derived antihypertensive hydrolysates (García-Tejedor, Padilla, Salom, Belloch, & Manzanares, 2013). With the aim of further characterize these non-conventional dairy yeasts as feasible GRAS (Generally Recognized As Safe) microorganisms for the production of antihypertensive peptides, here we screen up to thirty-three of these strains on the basis of their ability to release previously identified casein-derived antihypertensive sequences (RYLGY, AYFYPEL, LHLPLP, HLPLP, VPP and IPP) obtained either by enzymatic hydrolysis or bacterial fermentation. In addition, the presence of extracellular caseinolytic activity in selected strains is evaluated. Finally, quantification of the aforementioned sequences and identification of new main casein-derived peptides in the selected yeast casein hydrolysates are

carried out, and their potential bioactivities in terms of ACE inhibition and other functional effects are discussed.

2. Materials and Methods

2.1. Materials

Casein (Promilk 85) was obtained from Ingredia (Arrax Cedex, France). ACE from porcine kidney, bicinchoninic acid protein assay kit, Hammarsten casein and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). Glucose was obtained from Panreac (Barcelona, Spain), bacteriological peptone was purchased from Cultimed (Barcelona, Spain) and yeast extract and agar were acquired from Pronadisa (Madrid, Spain). The fluorogenic substrate o-aminobenzoyl-Gly-p-nitro-Phe-Pro was provided by Bachem Feinchemikalien (Bubendorf, Switzerland). Coomassie Brilliant Blue G-250, 30% acrylamide/bis solution 29:1 and Precision Plus Protein Dual Color standards were obtained from Bio-Rad Laboratories (Hercules, CA, USA).

2.2. Yeast strains and growth conditions

Thirty-three yeast strains belonging to *D. hansenii* (Dh1-Dh23), *K. lactis* (Kl1-Kl8) and *K. marxianus* (Km1-Km2) species isolated from artisanal ewes' and goats' milk cheeses (Padilla, Manzanares & Belloch, 2014) were used in this study. Yeast strains were maintained on GPYA medium (2% glucose, 0.5% peptone, 0.5% yeast extract and 2% agar, pH 5.5).

2.3. Preparation of casein hydrolysates

Stock solution of casein was sterilized by autoclaving at 121°C, 15 min. Casein medium (2% casein, 2% glucose) was inoculated with 10⁶ cells/ml from pre-cultured strains on GPY (GPYA without agar) and incubated at 28°C and 100 rpm in an orbital shaker for 6 days. At the end of the incubation period yeast cells were sedimented by centrifugation (3220 x g, 10 min), and the supernatants ultrafiltered using Amicon Ultra 10K centrifugal filter devices (Millipore Corporation, Billerica, MA, USA). Supernatant ultrafiltrates enriched in peptides of molecular weight lower than 10 kDa were considered as casein hydrolysates (CSHs). Supernatant concentrates (> 10 kDa) were used for zymography experiments as specified further.

Protein concentration was estimated by the bicinchoninic acid method using bovine serum albumin as standard (Ruiz-Giménez et al., 2012).

2.4. *In vitro* assay of ACE-inhibitory activity

In vitro ACE-inhibitory activity of CSHs was measured using the fluorescent method described by Sentandreu & Toldrá (2006) based on the hydrolysis of the internally quenched fluorescent substrate o-aminobenzoyl-Gly-p-nitro-Phe-Pro by the action of ACE.

The IC₅₀ value of CSHs was defined as the protein concentration required to inhibit 50% of the ACE activity, and the value for each experiment was estimated by non-linear regression of the experimental data to a four-parameter logistic curve using the software package SigmaPlot v 10.0 (SPSS Inc., Chicago, IL, USA).

2.5. Casein zymography

Zymography was performed according to Folio, Ritt, Alexandre, & Remize (2008) with minor modifications. Briefly, samples (casein supernatant concentrates) in loading buffer without β -mercaptoethanol were loaded onto a 10% (w/v) SDS/PAGE gel (Laemmli, 1970) co-polymerized with 0.12% (w/v) Hammarsten casein. After electrophoresis and brief washing in ultrapure water, the gel was washed for 2 h on a rotary shaker with 2.5% (v/v) Triton X-100. Afterwards the gel was washed twice with reaction buffer (50 mM citrate phosphate buffer, pH 6) and then incubated overnight in reaction buffer at room temperature. The gel was stained with 0.1% (w/v) Coomassie Brilliant Blue G-250 for 1 h and then de-stained. Protease activity bands were visualized as white bands on a dark blue background.

2.6. Synthesis and quantification of peptides HLPLP and LHLPLP

The peptides HLPLP f(134-138) and LHLPLP f(133-138) from β -casein, were synthesized in-house using the fluorenyl-methoxy-carbonyl chloride (Fmoc) solid-phase method with a 431A peptide synthesizer (Applied Biosystems Inc. Überlingen, Germany). Peptide purity was calculated by reversed phase high performance liquid chromatography-UV and mass spectrometry (RP-HPLC-UV-MS).

Six-point calibration curves of the pure peptides HLPLP and LHLPLP (from 1.95 to 62.5 $\mu\text{g/mL}$) were prepared in Milli-Q deionized water. Duplicate injections were done for each point on the calibration curve. The quantification of the peptides in each CSH was performed by the extraction of their characteristic ions.

175 *2.7. Peptide sequencing by reversed phase-high-performance liquid*
176 *chromatography tandem mass spectrometry (RP-HPLC-MS/MS).*

177 The analyses of CSHs by RP-HPLC-MS/MS were performed on an
178 Agilent HPLC (Agilent Technologies, Waldbronn, Germany) system followed by
179 on-line MS/MS analysis on a quadrupole ion trap instrument (Esquire 3000,
180 Bruker Daltonik GmbH, Bremen, Germany) as previously described (Sánchez-
181 Rivera, Diezhandino, Gómez-Ruiz, Fresno, Miralles, & Recio, 2014).
182 Chromatographic separations were performed with a Mediterranea Sea₁₈ 150
183 mm × 2.1 mm column (Teknokroma, Barcelona, Spain). The flow rate was 0.2
184 mL/min and the injection volume 50 µL. Peptides were eluted with a linear
185 gradient from 0% to 45% of solvent B (acetonitrile:formic acid 0.1%) and 55%
186 solvent A (water:formic acid 0.1%) in 120 min. The target mass was set at
187 *mass-to-charge* ratio (*m/z*) 750, and the mass acquisition ranged from 200 to
188 2000 *m/z*.

189
190 *2.8. Statistical data analysis*

191 IC₅₀ values of CSHs were compared using Student's t-test. Differences
192 with *P*-values < 0.05 were considered significant. Data statistical analysis was
193 performed using the GraphPad Prism 4 software (GraphPad Software Inc, La
194 Jolla, CA, USA).

195
196 **3. Results and discussion**

197 Non-Saccharomyces yeasts have been utilized as industrial organisms
198 for a variety of biotechnological roles since numerous and diverse biological
199 activities make them promising candidates for a wide range of applications not

limited to the food sector. They are increasingly being used as hosts for expression of proteins, biocatalysts and multienzyme pathways for the synthesis of fine chemicals and small molecular weight compounds of medicinal and nutritional importance (Jonhson, 2013).

In the present study, 33 CSHs generated by *D. hansenii* (CSH Dh1-Dh23) *K. lactis* (CSH KI1-KI8) and *K. marxianus* (CSH Km1-Km2) strains isolated from artisanal cheeses (Padilla et al., 2014) were analysed by HPLC-MS/MS to search for the previously identified antihypertensive sequences RYLGY, AYFYPEL, LHLPLP, HLPLP, VPP and/or IPP. Peptides RYLGY, AYFYPEL, VPP and IPP were not identified in any of the CSHs. However, peptide HLPLP and its precursor sequence LHLPLP were released from casein after growth of *D. hansenii* strains Dh1 and Dh14. Figure 1 shows the UV chromatogram and the extracted ions corresponding to both peptides.

Quantification of HLPLP and LHLPLP in CSH Dh1 and Dh14 was carried out using linear regressions obtained by HPLC-MS/MS. As shown in Table 1, highest total content was obtained in CSH Dh14. However both hydrolysates contained similar quantities of the pentapeptide. These antihypertensive peptides represented approximately 6 and 10% of total peptide content in Dh1 and Dh14 hydrolysates, respectively. No significant differences were found between the ACE inhibitory potencies of both *D. hansenii* CSHs, which showed IC₅₀ values of 13.6 ± 1.8 µg/mL (Dh1) and 17.5 ± 2.1 µg/mL (Dh14). ACE inhibition was slightly higher than the previously reported values of milk fermented by *E. faecalis* (34-59 µg/mL; Muguerza et al., 2006) but lower than that showed by a pepsin casein hydrolysate (5.68 µg/mL; Contreras et al., 2009).

During milk fermentation, proteolysis by LAB has been studied in detail. The key enzyme in this process is a cell-envelope proteinase involved in the first step of casein degradation (Savijoki, Ingmer, & Varmanen, 2006). By contrast, although yeast caseinolytic activity is well documented few reports deal with the characterization of the extracellular proteolytic system. With regards to *D. hansenii*, Kumura, Takagaki, Sone, Tsukahara, Tanaka, & Shimazaki (2002) reported that a cell-wall associated protease acted on β -casein whereas α_S -casein degradation was attributed to an intracellular protease released by cell lysis. However, extracellular proteolytic activity on both α_S - and β -casein for *D. hansenii* strains isolated from cheese has been described (Gardini et al., 2006). Here we conducted a casein zymography to detect extracellular proteases in the supernatants of the two selected *D. hansenii* strains. On casein substrate, extracellular proteolytic activities were detected (Figure 2). Analysis of Dh14 supernatant revealed the presence of one hydrolysis band which corresponded to a molecular mass of about 50 kDa. The same band was observed in Dh1 supernatant, in which a second band of slightly higher molecular mass was also detected. Band intensities for both supernatants were not related to the amount of total protein loaded (Dh1, 4.8 μ g; Dh14, 4.4 μ g). Although increasing amounts of Dh14 total protein were loaded onto SDS/PAGE gels for protease detection no other clear activity bands were observed (data not shown). These results might suggest a different proteolytic profile for both strains in the conditions tested. Future work should include purification of the extracellular *D. hansenii* enzymes and determination of its biochemical properties.

Over the last two decades, a large number of casein-derived peptides obtained by fermentation and enzymatic hydrolysis with ACE-inhibitory potency and proven antihypertensive effects in SHR_s have been identified (Martínez-Maqueda et al., 2012). Although LHLPLP and HLPLP might contribute to the inhibitory potency of *D. hansenii* CSHs, other sequences generated from casein hydrolysis might also be responsible for the observed inhibitory effects. This prompted us to carry out a complete analysis of CSH Dh1 and Dh14 by HPLC-MS/MS, which allowed the identification of 35 and 46 additional peptides (Table 2 and 3), respectively. The most abundant peptides in each hydrolysate are given in bold. As can be seen in Table 2, most of the peptides present in CSH Dh1, derived from hydrolysis of α_{S1} -CN (15 fragments) and β -CN (14 fragments) whereas only 6 and 2 peptides corresponded to α_{S2} -CN and κ -CN fragments, respectively. By contrast, in CSH Dh14 most of the peptides (37 fragments) derived from β -CN. In addition 9 fragments from α_{S1} -CN and 2 from κ -CN were also identified, while none of the fragments derived from α_{S2} -CN. Finally, only 11 of the peptides identified, including the two antihypertensive sequences LHLPLP and HLPLP, were common for both *D. hansenii* strains, which indicates strain-specific proteolysis in accordance with the results obtained in the zymogram and also as described for the *Streptococcus thermophilus* cell envelope proteinase (Miclo et al., 2012).

Sequences LHLPLP and HLPLP were also included in several longer fragments identified in CSH Dh14, such as LHLPLPL, HLPLPL and LHLPLPLLQS. The heptapeptide LHLPLPL, with an IC_{50} value of $425 \pm 44 \mu M$ against ACE, was also identified in *E. faecalis* fermented milk (Quirós et al., 2007). Another peptide (β -CN f(58-76), LVYPFPGPIPNSLPQNIPP; $IC_{50} = 5.2 \pm$

0.3 μ M) identified in *E. faecalis* fermented milk caused also significant decrease of the systolic blood pressure in SHR_s (Quirós et al., 2007). Part of this sequence (VYPFP) was also identified in both *D. hansenii* CSH_s. On the other hand, although neither VPP nor IPP were found in *D. hansenii* CSH_s, sequences containing the latter at the N-terminal end were identified (IPPL, IPPLTQ, IPPLTQT and IPPLTQTP). Whether these newly identified sequences could also contribute to the ACE-inhibitory activity of CSH Dh1 and Dh14 deserves further characterization studies. Moreover, the role of gastrointestinal digestion to generate active fragments from longer sequences should also be taken into account, although degradation of active fragments can also occur.

More than 60% of the identified sequences have at least one Pro residue. Particularly, Pro in the C-terminal and antepenultimate position has been recognised as one of the most favourable residues for peptide binding to the active site of ACE (Rohrbach, Williams, & Rolstad, 1981). Interestingly, in addition to the antihypertensive sequences LHLPLP and HLPLP, peptides VAPFP, DIPNP, VYPFP, FSDIPNP and GPVRPFP with a Pro residue in both positions might be promising candidates to inhibit ACE activity. Finally, some other sequences identified in CSH Dh1 and Dh14 were previously characterized as ACE-inhibitory and antioxidant peptides (sequence YQEP in Dh1; Silva, Pihlanto, & Malcata, 2006) or having lipoxygenase inhibitory properties (VLPVPQK in Dh1; Rival, Fornaroli, Boeriu, & Wichers, 2001). Also some sequences identified here share structure homology with fragments able to inhibit prolyl-peptidases of human colon cells (FVAPFPE, VAPPFP and VAPFPE in Dh1; Juillerat-Jeanneret, Robert, & Juillerat, 2011) or having immune-modulating and ACE-inhibitory properties (GPVRGPFPI and

GPVRGPFP in Dh14; Hayes, Stanton, FitzGerald, & Ross, 2007; Hernández-Ledesma, Amigo, Ramos, & Recio, 2004). The multifunctional properties and the resulting health benefits of dairy protein hydrolysates have been pointed out by several studies (reviewed in Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo, & Recio, 2014). To what extent *D. hansenii* CSHs might exert different functional effects requires further research.

D. hansenii is a highly heterogeneous, and thus versatile, species which represents an attractive target for fundamental and applied biotechnological research (Johnson, 2013; Hatoum et al., 2013). Our results point to dairy *D. hansenii* strains Dh1 and Dh14 as feasible GRAS microorganisms with strain-specific caseinolytic systems for the production of the antihypertensive sequences HLPLPL and HLPLP. These two peptides could at least in part be responsible for the ACE-inhibitory properties of both *D. hansenii* CSHs, although data reported here suggest that other potential bioactive sequences are also produced. *D. hansenii*, one of the predominant yeast species in all type of cheeses, plays important roles in cheese making and further investigation is needed to exploit its biotechnological potential.

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777.435	776.428	776.407	α_{S1} -CN f(167-173)	VPLGTQY
581.257	580.25	580.286	α_{S1} -CN f(169-173)	LGTQY
555.344	554.337	554.27	α_{S1} -CN f(181-185)	DIPNP
519.164	518.156	518.234	α_{S1} -CN f(186-190)	IGSEN
602.321	601.313	601.253	α_{S2} -CN f(4-8)	MEHVS
1000.532	999.525	999.539	α_{S2} -CN f(100-108)	YQGPIVLNP
837.459	836.452	836.476	α_{S2} -CN f(101-108)	QGPIVLNP
698.394	697.386	697.401	α_{S2} -CN f(116-122)	AVPITPT
528.248	527.24	527.296	α_{S2} -CN f(118-122)	PITPT
720.417	719.409	719.422	α_{S2} -CN f(198-203)	TKVIPY
628.27	627.263	627.323	β -CN f(6-11)	LNVPGE
748.265	747.258	747.304	β -CN f(38-43)	QQQTED
622.309	621.302	621.316	β -CN f(59-63)	VYPFP
439.026	438.018	438.284	β -CN f(74-77)	IPPL
668.38	667.373	667.39	β -CN f(74-79)	IPPLTQ
732.347	731.34	731.385	β -CN f(113-118)	KYPVEP
690.282	689.275	689.323	β -CN f(127-132)	LTDVEN
577.164	576.157	576.239	β -CN f(128-132)	TDVEN
689.449	688.442	688.427	β -CN f(133-138)	LHLPLP
576.304	575.297	575.343	β -CN f(134-138)	HLPLP
649.265	648.258	648.28	β -CN f(144-148)	MHQPH
805.351	804.343	804.384	β -CN f(155-161)	VMFPPQS
780.457	779.45	779.491	β -CN f(170-176)	VLPVPQK
536.148	535.14	535.228	β -CN f(193-196)	YQEP
633.35	632.343	632.353	k-CN f(26-30)	IPIQY
663.318	662.311	662.339	k-CN f(43-47)	YQQKP

0 ^aCharge of precursor ion: 1
1 ^bCalculated monoisotopic mass.
2 ^cMain peptides are labeled in bold.
3 Casein: CN
4

Table 3. Identification of peptides contained in *D. hansenii* casein hydrolysate (CSH) Dh14.

Ion for MS/MS (m/z) ^a	Observed mass ^b	Theoretical mass	Protein fragment	Identified sequence ^c
805.399	805.378	805.401	α_{S1} -CN f(24-30)	FVAPFPE
676.407	675.400	675.359	α_{S1} -CN f(29-34)	PEVFGK
707.446	706.439	706.401	α_{S1} -CN f(31-36)	VFGKEK
763.321	762.322	762.358	α_{S1} -CN f(54-59)	MEDIKQ
732.394	631.328	631.318	α_{S1} -CN f(55-59)	EDIKQ
903.472	902.465	902.453	α_{S1} -CN f(135-142)	MIGVQNQL
517.171	516.164	516.233	α_{S1} -CN f(160-164)	PSGAW
789.371	788.634	788.370	α_{S1} -CN f(179-185)	FSDIPNP
730.385	729.314	729.329	α_{S1} -CN f(184-190)	NPIGSEN
675.257	674.322	674.324	β -CN f(1-5)	RELEE
649.284	627.306	627.323	β -CN f(6-11)	LNVPGE
633.295	632.288	632.277	β -CN f(38-42)	QQQTE
748.284	747.277	747.304	β -CN f(38-43)	QQQTED
875.371	876.305	876.346	β -CN f(38-44)	QQQTEDE
632.355	631.335	631.318	β -CN f(44-48)	ELQDK
681.303	680.296	680.313	β -CN f(52-57)	FAQTQS
622.309	621.302	621.316	β -CN f(59-63)	VYPFP
668.416	667.409	667.390	β -CN f(74-79)	IPPLTQ
769.342	768.386	768.438	β -CN f(74-80)	IPPLTQT
866.472	865.465	865.491	β -CN f(74-81)	IPPLTQTP
753.385	752.378	752.407	β -CN f(75-81)	PPLTQTP
689.313	688.395	688.412	β -CN f(95-100)	VSKVKE
760.456	759.449	759.449	β -CN f(95-101)	VSKVKEA
577.327	576.267	576.294	β -CN f(98-102)	VKEAM
651.384	650.376	650.343	β -CN f(111-115)	FPKYF
732.274	731.364	731.385	β -CN f(113-118)	KYPVEP
611.329	610.267	610.260	β -CN f(119-123)	FTESQ
698.269	697.262	697.292	β -CN f(119-124)	FTESQS
904.446	903.439	903.455	β -CN f(125-132)	LTLTDVEN
689.313	688.392	688.427	β -CN f(133-138)	LHLPLP
802.505	801.498	801.511	β -CN f(133-139)	LHLPLPL
1130.667	1129.660	1129.686	β -CN f(133-142)	LHLPLPLLQS
576.334	575.327	575.343	β -CN f(134-138)	HLPLP
793.521	792.514	792.511	β -CN f(135-141)	LPLPLLQ
880.515	879.508	879.543	β -CN f(135-142)	LPLPLLQS
670.420	669.413	669.406	β -CN f(137-142)	LPLLQS
533.184	532.177	532.265	β -CN f(140-143)	LQSW
649.329	648.277	648.280	β -CN f(144-148)	MHQPH
805.402	804.348	804.384	β -CN f(155-161)	VMFPQQS
649.327	648.320	648.344	β -CN f(164-169)	SLSQSK
543.270	542.262	542.343	β -CN f(168-172)	SKVLP
559.253	558.246	558.284	β -CN f(185-189)	MPIQA
762.385	761.378	761.396	β -CN f(191-196)	LLYQEP
649.318	648.310	648.312	β -CN f(192-196)	LYQEP
826.445	825.438	825.450	β -CN f(199-206)	GPVRGPF
939.500	938.493	938.534	β -CN f(199-207)	GPVRGPFPI
658.370	657.363	657.370	k-CN f(119-124)	IPTINT
757.362	756.355	756.365	k-CN f(155-161)	SPPEINT

^aCharge of precursor ion: 1

^bCalculated monoisotopic mass.

^cMain peptides are labeled in bold.

CN: casein

Figure captions

Figure 1. UV chromatogram of the casein hydrolysate Dh1 (A) and Dh14 (B).
Extracted Ion chromatogram (EIC) of the peptides LHLPLP and HLPLP in Dh1
(C) and Dh14 (D). Fragmentation pattern of LHLPLP (E) and of HLPLP (F), with
mass-to-charge ratios (m/z) of 689.5 and 576.3, respectively.

Figure 2. Casein zymography of *D. hansenii* Dh1 and Dh14 extracellular
proteolytic activities. Lane M, molecular weight markers (Precision Plus Protein
Dual Color standards, Bio-Rad); lane 1, Dh1 culture supernatant concentrate
(4.8 μg); lane 2, Dh14 culture supernatant concentrate (4.4 μg).

Figure 1

Figure 2

Figure 2 García-Tejedor et al., 2014