# Proposing Sequences for Peptides Derived from Whey Fermentation with Potential Bioactive Sites

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## ABSTRACT

In fed-batch fermentation by *Kluyveromyces marxianus* var. *marxianus*, whey-soluble proteins were converted into oligopeptides. To assess whether bioactive peptides could be produced during whey fermentation, *K. marxianus* was cultured in batch in deproteinized media containing 5 or 15% (wt/vol) dehydrated whey for 20 h and then was in fed-batch mode for 50 h. After harvesting the biomass (25,000 × *g*, 15 min), at 6-h intervals, the wort was analyzed to determine protein consumption and oligopeptide production by HPLC. The proteins in the wort showed an oscillatory degradation with a constant increase in the production of oligopeptides. Four major peaks were collected and were analyzed by API mass spectroscopy. Sequences of fermented peptides were compared with sequences of known bioactive peptides. On the basis of their molecular weights, two amino acid sequences were proposed. The presence of sites containing the peptide sequence of β-lactorphin (YLLF) suggests that these oligopeptides may have antihypertensive properties.
(**Key words**: bioactive peptides, *Kluyveromyces marxianus*, whey fermentation, antihypertensive properties)

**Abbreviation key**: **ACE** = angiotensin-I-converting enzyme, **MS** = mass spectrometry, **MW** = molecular weight, **OP** = oligopeptides, **YE** = yeast extract.

## INTRODUCTION

Whey protein fractions are nutritionally equal to or superior to commonly available food proteins such as casein, egg albumin, and soy. These fractions are considered to be of nutritive and physiological importance when administered orally. Whey contains major proteins such as β-LG, α-LA, BSA, and Ig (IgG, IgA, and IgM). They represent 50, 20, 12, and 8%, respectively, of total whey protein in bovine milk. The β-LG, is normally present as a dimer, consists of two β-LGA, two β-LGB, or one β-LGA and one β-LGB, and it may be involved in vitamin A transport. The α-LA strongly binds Ca with a dissociation rate constant ($K_d$) of 10 to 12 *M*. It participates in lactose synthesis. The BSA and immunoglobulins are identical to their blood serum counterparts and probably pass into milk with blood serum. The BSA in milk serves no known function. Immunoglobulins, which are antibodies, are particularly high in colostrum and mastitic milk. Whey also contains minor proteins such as lactoperoxidase, lactoferrin, glycomacropeptide, and proteose peptones. Lactoferrins are biologically active proteins. Proteose peptones are a mixture of heat-stable, acid-soluble (pH 4.6) polypeptide fragments of caseins (f 1–18, f 29–105, f 29–107, f 1–105, and f 1–107), which arise from the proteolysis of caseins by indigenous milk enzymes. Glycomacropeptides are the sequence of κ-CN (residues f 105–169) generated by the hydrolysis of κ-CN by chymosin during cheese processing (6, 12, 15).

Many workers have suggested that fermented dairy products exhibit antimutagenic, antihypertensive, and antitumor activities and antibacterial properties. Most of these studies have been carried out using α-, β-, and κ-CN derivatives (2, 3, 7, 8, 10, 13, 18) and lactoferrin (5, 14, 16). However, there have been some reports suggesting that short peptides obtained from fermented whey proteins are among the most potent pharmacologically active agents (9, 11, 17). Antimutagenic activity of whey fermented by the proteolytic variant ($Prt^+$) of *Lactobacillus helveticus* has been demonstrated in vivo against a mutagen, 4-nitro-quinoline-N′-oxide, in *Salmonella typhimurium*. In vivo carcinogenesis has been shown (9) to be related to in vitro mutagenesis. α-Lactorphin [YGLF, α-LA (f 50–53)], a tetrapeptide originating from the proteolysis of bovine α-LA; β-lactorphin [YLLF, β-LG (f 102–105)]; and β-lactotensin [HIRL, β-LG (f 146–149)], tetrapeptides

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originating from the proteolysis of bovine $\beta$-LG, have been reported to show angiotensin-I-converting enzyme (**ACE**) inhibitory activity (11). Among these tetrapeptides, $\beta$-lactorphin showed the highest ACE inhibitory activity, $IC_{50}$ = 171.8 $\mu M$ (i.e., 171.8 $\mu M$ of this peptide causes 50% inhibition of ACE using hippuryl-histidyl-leucine as substrate). A dipeptide related to $\beta$-lactorphin (YL) was reported to be even more potent: $IC_{50}$ = 122 $\mu M$. These peptides are considered to be of nutritive and physiological importance and can be active following oral administration.

Belem and Lee (1) demonstrated that fed-batch fermentation of whey by *Kluyveromyces marxianus* var. *marxianus* produced oligopeptides (**OP**). The purpose of this work was to select some of these oligopeptides, propose a sequence of amino acids for these compounds, and compare them with known sequences of bioactive peptides. The fermentation kinetics of whey proteins were also studied.

## MATERIALS AND METHODS

### Microorganism

*Kluyveromyces marxianus* var. *marxianus* ATCC 28244, formerly named *Kluyveromyces fragilis*, was used in this experiment. The strain was propagated at 30°C in 1% (wt/vol) yeast extract (**YE**), 2% peptone, and 2% dextrose (Sigma Chemical Co., St. Louis, MO). Culture revival was achieved in one subculturing before inoculation into the fermentor.

### Medium and Inoculum Preparation

Two different media were used in this experiment. Medium A was composed of 5% (wt/vol, dry weight) dehydrated whey (Fromages Saputo, St. Hyacinthe, PQ, Canada) supplemented with 0.5% (wt/vol) YE (Difco Laboratories, Detroit, MI), 0.4% (wt/vol) $(NH_4)_2SO_4$ (American Chemicals, Ltd.), 0.2% (wt/vol) $KH_2PO_4$ (Fischer Co., Fair Lawn, NJ), and 1 ml/L of antifoam B (Sigma Chemical Co.). The pH was adjusted to 4.5 with 0.05 $M$ $H_2SO_4$. Medium B was composed of 15% (wt/vol) dehydrated whey with 0.5% (wt/vol) YE, 0.4% (wt/vol) $(NH_4)_2SO_4$, and 0.2% (wt/vol) $KH_2PO_4$. Inocula containing medium A (200 ml) were grown at pH 4.5 in Erlenmeyer flasks (500 ml) with agitation (85 rpm) for 20 h. Both media were heated at 115°C (pH 4.5) for 5 min and were centrifuged (10,000 $\times$ $g$, 15 min, 5°C) to remove insoluble denaturated proteins ($\alpha$-LG, BSA, and IG). Before inoculation, the media were sterilized at 121°C (pH 4.5) for 15 min.

### Fermentation

Batch cultivation was carried out in a 20-L fermentor (Bioengineering AG 2) filled with 5 L of medium A at 30°C (pH 4.5) with agitation (350 rpm) and aeration (2 vvm). Before the end of the log phase (20 h of fermentation), continuous feeding of medium B at flow rate of 180 ml/h was started. Growth was monitored by sampling 30 ml at 6 to 8-h intervals and by measuring cell numbers by direct microscopic counting (cells per milliliter) for 70 h. Yeast cell density ($\delta$) was determined by fermenting whey permeate (5%, wt/vol) in batch and by measuring both cell growth (cells per milliliter) and biomass (grams per liter, dry weight). Lactose consumption and ethanol production (grams per liter) were assayed using enzymatic lactose/D-galactose and ethanol determination kits, respectively (Boehringer Manheim, Germany). Protein consumption (grams per liter) was determined by BCA protein assay (Pierce) and measured spectrophotometrically at 562 nm. Dissolved oxygen (percentage, vol/vol) was measured using a probe attached to the fermentor. Dissolved oxygen of 100% (vol/vol) was defined as the amount of oxygen detected by the probe when 5.0 L of sterile medium containing 5% (wt/vol) dehydrated whey was under 2 vvm aeration and 350 rpm agitation at pH 4.5 and 30°C. After fermentation, yeast cells were harvested (25,000 $\times$ $g$, 15 min, 5°C) by a centrifuge (Sharples T-1P; Alfa-Laval Group, Warminster, PA). The supernatant (wort) was then analyzed by HPLC to determine the presence of OP released during whey fermentation.

### HPLC

An HPLC system (600 E system controller, a U6K injector, a 486 tunable absorbance UV detector, and a Millennium 2010 chromatography manager; Waters, Milford, MA) was used to detect the OP. To detect OP, 120-$\mu$l aliquots of the harvested wort were injected on a DeltaPak $C_{18}$ column (100 Å, 30 mm $\times$ 150 mm). All samples were filtered through a 0.45-$\mu$m high binding protein filter (Cole Parmer) before injection. Samples were eluted with a linear gradient from 85% (vol/vol) to 0% of solution A and from 15 to 100% (vol/vol) of solution B at a flow rate of 0.7 ml/min. Solution A was water (18 ohm), and acetonitrile (95/5, vol/vol) and 0.1% (vol/vol) trifluoroacetic acid. Solution B was water and acetonitrile (60/40, vol/vol) and 0.1% trifluoroacetic acid. Solution B (15%, vol/vol) was initially added isocratically for 10 min and then from 15 to 40% in a linear gradient for 5 min, from 40 to 60% in a linear gradient for 15 min, from 60 to 100% in a linear gradient

for 15 min, from 100 to 15% for 10 min, and, finally, 15% isocratically for 15 min. The absorbance was measured at 215 nm. After elution under the same conditions, four peaks detected by HPLC (peaks a, b, c, and d) were selected, and the fractions corresponding to these peaks from 10 runs were pooled using a fraction collector (Waters). The collected samples were then analyzed by a mass spectrometer to determine molecular weights (**MW**).

### Mass Spectrometry

Mass spectra were obtained in the positive ion mode on a triple stage mass spectrometer (model API-III; Sciex, Toronto, Canada). Samples (concentrations ranging from 10 to 100 $\mu M$) were dissolved in 10% (vol/vol) acetic acid and were infused through a stainless steel capillary (100 $\mu M$ ID). A stream of air was introduced to assist in the formation of submicron droplets (pneumatic nebulization) (4). These droplets were evaporated at the interface by nitrogen gas to produce highly charged ions, which were detected by the analyzer. Calibration of the system was performed with ammonium adduct ions of polypropylene glycol with a known mass to charge ratio (m/z) throughout the range of 0 to 2470 atomic mass units. Mass charges were used throughout the range of the instrument (0 to 2470 atomic mass units). Instrument tuning and data acquisition and processing were controlled by a Macintosh II computer with all software provided by the instrument manufacturer. The software included simple algorithms to correlate the charges produced by these compounds to their MW. After determining the MW of the different compound fragments, sequences of amino acids for peptides a, b, c, and d were proposed. The accuracy of amino acid sequences that were proposed was verified by determining the deviations of the MW of the different fragments obtained by mass spectrometry (**MS**) from the MW of the proposed peptides. Maximum errors were expressed as the percentage (wt/wt) of the deviation (maximum) of the MW that were obtained by MS divided by the MW of the proposed peptides.

## RESULTS

Figure 1 shows the kinetics of lactose consumption, biomass and ethanol production, and the relative concentration of dissolved oxygen in the wort. The production of ethanol during fermentation was below 1.0 g/L, which indicates that the high aeration (2

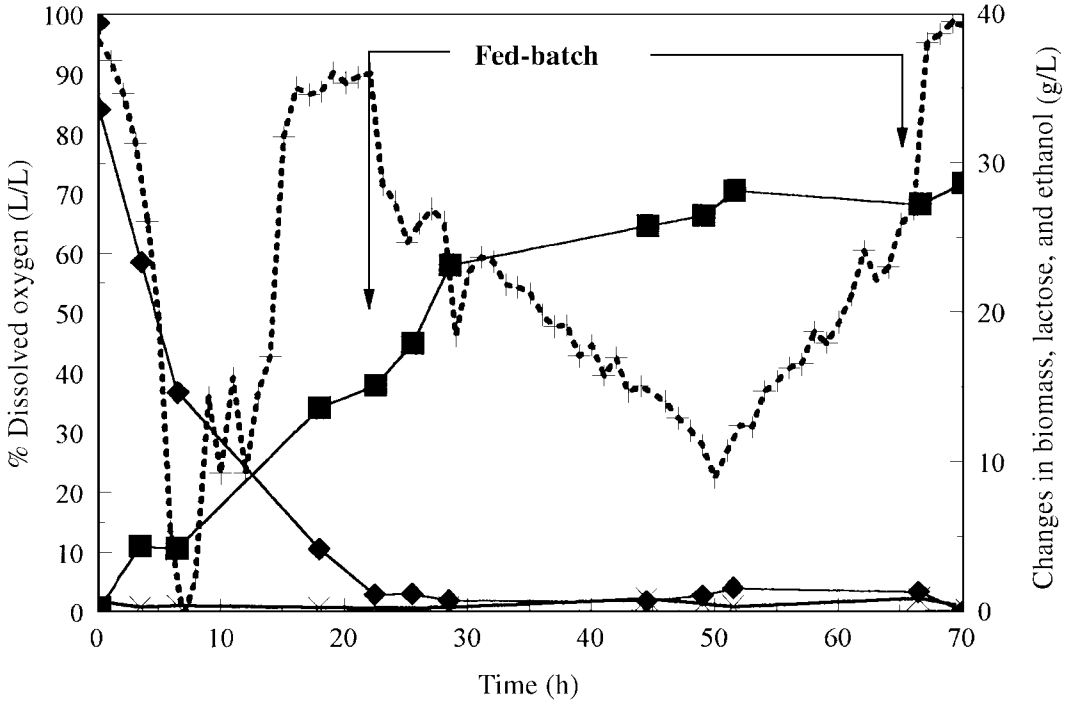


Figure 1. Kinetics of fed-batch fermentation by *Kluyveromyces marxianus* var. *marxianus* grown on whey are shown by lactose consumption (♦), biomass production (■), and ethanol production (×) and by the relative concentration of oxygen dissolved in the wort (+).

vvm) reduced the glycolytic metabolism and that whey fermentation was mostly accomplished by the oxidative metabolism of *Kluyveromyces marxianus*. At the beginning of the log phase of the microbial growth (8 h) in the batch mode of fermentation, almost all of the oxygen had been consumed by the yeast (below 15% vol/vol of dissolved oxygen). After 18 h of fermentation, the stationary phase started, but lactose concentration (below 5 g/L) instead of oxygen was the limiting factor for the microbial growth. Immediately after the beginning of the fed-batch stage of fermentation (22 h), the concentration of dissolved oxygen in the medium sharply decreased from 90 to 60% (vol/vol). After 50 h of growth, a stationary phase was reached, and oxygen consumption decreased. The concentration of dissolved oxygen increased constantly from below 10% to more than 90% (vol/vol). Aging and mortality were higher than reproduction after 50 to 70 h of fermentation.

Figure 2 shows the oscillatory profile of protein degradation in the wort during fermentation. In the batch mode of fermentation, the concentration of soluble proteins decreased from 6.0 to 2.5 g/L. Immediately after starting the fed-batch fermentation, soluble proteins in the wort were accumulated, and they increased from 2.5 to 3.8 g/L. When the stationary phase was attained after 50 h of fermentation, the concentration of proteins in the medium started to decrease again from 5.5 to 3.5 g/L.

Figure 3 shows a chromatogram of the supernatant after harvesting the biomass. The peptide elution profile at 0 h shows the peptides that were detected in the sterile medium [5% (wt/vol) of dehydrated whey]. The sterile medium containing 15% (wt/vol) dehydrated whey presented a very similar profile (data not shown), which indicated that any changes in the profile of peptides resulted from the partial hydrolysis of soluble proteins (lactoglobulin) during fermentation and not from the addition of concentrated medium in fed-batch fermentation. The chromatograms after 18 h of batch fermentation and after 45 to 67 h of fed-batch fermentation showed two types of hydrophilic and hydrophobic peptides that were eluted before and after 35 min.

The production of many hydrophilic peptides (first half) could be observed in both batch and fed-batch fermentations. The increase in the peak heights and areas indicates an increase in the concentration of these peptides in the medium. Two peaks were selectively collected and analyzed by MS: peak a (retention time of 23 min) and peak b (retention time of 34.5 min). However, in the second half of the chromatogram, the heights and areas of some peaks such as c (retention time of 37.5 min) decreased after 48 h

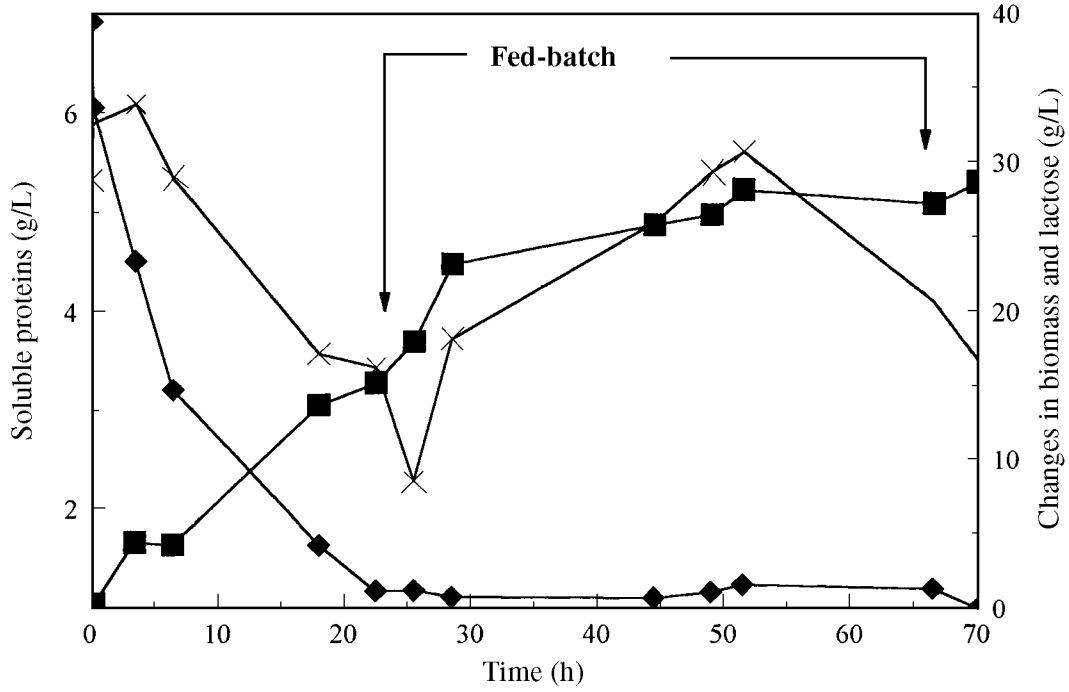


Figure 2. Consumption and production of soluble (X) proteins in a fed-batch fermentation by *Kluyveromyces marxianus* var. *marxianus* grown on whey. Biomass production (■); lactose consumption (♦).

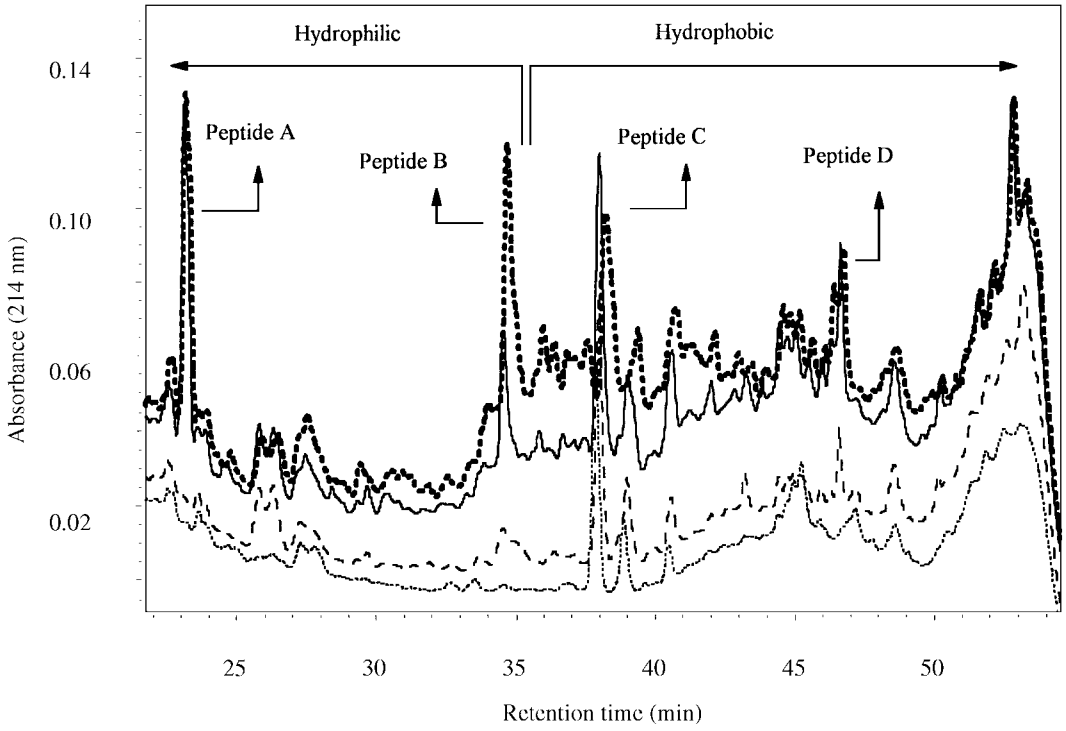


Figure 3. Production of oligopeptides during whey fermentation for 0 h (· · ·), 18 h (– –), 45 h (—), and 67 h (●●●) by *Kluyveromyces marxianus* var. *marxianus*.

of fermentation, and others such as peak d (retention time of 46.5 min) increased during the entire fermentation. Peaks c and d were also collected and analyzed by MS. The profile of peak c in the fed-batch fermentation was similar to that of proteins shown in Figure 2.

Tables 1 and 2 show the sequence of amino acids for the fragments obtained by MS. In Table 1, peaks a and b were analyzed. In Table 2, peaks c and d were analyzed. The sequence of amino acids, obtained after fragmentation by API-MS, were compared with sequences of peptides of $\alpha$-LA and $\beta$-LG. Two considerations were taken to propose the sequence of amino acids. Because $\beta$-LG is the major protein component of whey and because it is less sensitive to heat treatment, all proposed sequences of amino acids that were obtained after fragmentation by API-MS were based on the sequences of both variants, $\beta$-LG A and $\beta$-LG B. Fragments of the main peptides are shown in bold (at least one of the fragments was the base peak, intensity = 100%), fragments of residual peptides are in italics, and bioactive sites are underlined. The MW of the peak fragments obtained by MS with intensities different from 100% did not match with the MW of peptides derived from $\alpha$-LA.

From Tables 1 and 2, the sequence of amino acids for peaks a, b, and d were proposed as follows: **R VY** for peak a; **TDYK KYLLFCMENS** or **DYK KYLLFCMENS A** for peak b; and **TDYK K** or **CMENS A** for peak d. Although peak b can be composed of amino acids in either the f 97–110 or f 98–111 positions from $\beta$-LG A or $\beta$-LG B, both proposed peptides contain the bioactive site YLLF ($\beta$-lactorphin) (11). As the area of peak b increased during fermentation, *Kluyveromyces marxianus* apparently was not able to use this peptide as a source of N or C for its metabolism. However, these peptides are much longer than the bioactive peptide YLLF (11). It is possible, thus, that the peptide configuration still hides this bioactive site. The increase of the area of peak d, **TDYK K** or **CMENS A** (f 97–101 or f 106–111) also suggests that, during proteolysis, *Kluyveromyces marxianus* cannot metabolize these peptides.

From Table 2, the sequence of amino acids for peak c was proposed as follows: **TQTMKGL DIQKVAGTWY SLAMAASDIS LDAQSAP** (f 4–38). This peptide contains none of the previously identified bioactive sites YLLF and HIRL ($\beta$-lactotensin). The decrease of the area of peak c after 48 h indicates that *K. marxianus* used whey OP as a source of either N or C at the final stages of fermentation. Residues of this peptide were also detected when analyzing peaks b, *QTMK* and *LIVTQTM* (Table 1), and d, *LIVTQTM*

TABLE 1. Sequence of amino acids for the fragments of peptides a and b after mass spectrometry.

| HPLC Peak | Ion mass of fragments | Intensity | Possible sequence of amino acids for fragments | MW[1] of peptide proposed | Maximum error | Amino acid position in β-LG |
|---|---|---|---|---|---|---|
| | | (% base of peak) | | | (%) | |
| a | 437.1 | 100 | **R VY**[2] | 437.52 | 0.02 | 40–42 |
| | 453 | 90 | R VY + O | 453.52 | 0.02 | 40–42 |
| | 416.9 | <10 | *P EGD*[3] | 417.4 | 0.45 | 50–53 |
| | 477 | <5 | R VY + K | 476.52 | 0.02 | 40–42 |
| | 359.1 | <4 | EGD + K | 359.38 | 0.13 | 51–53 |
| | 831.5 | <4 | RTPEVDD | 831.86 | 0.09 | 124–130 |
| b | 1654.7 | 100 | **DYK KYLLFCMENS**[2] | 1654.94 | 0.05 | 98–110 |
| | | | DIQKVAGTWY SLAMA | 1654.92 | 0.05 | 11–25 |
| | 655.2 | 56 | **TDYK K**[2] | 654.74 | 0.15 | 97–101 |
| | | | **CMENS A**[2] | 654.73 | 0.15 | 106–111 |
| | 1677.1 | 18 | DYK KYLLFCMENS + K | 1693.94 | 0.05 | 98–110 |
| | | | DIQKVAGTWY SLAMA + K | 1693.92 | 0.05 | 11–25 |
| | 806.2 | 45 | *LIVTQTM*[3] | 806.01 | 0.05 | 1–7 |
| | 502.7 | 48 | VFKI | 507.67 | 0.19 | 81–84 |
| | | | *QTMK*[3] | 507.63 | 0.18 | 5–8 |
| | 1920.4 | 12 | ASDIS LLDAQSAPLR VYV | 1919.19 | 0.13 | 26–43 |
| | 2287.9 | 4 | RTPEVDD EALEKFDKALKAL | 2289.59 | 0.16 | 124–143 |

[1]Molecular weight.

[2]Fragments of the main peptide (in bold). Proposed sequence of amino acids for peptide a, **R VY**. Proposed sequence of amino acids for peptide b, **tDYK KYLLFCMENSa**.

[3]Residual peptides (in italics). Proposed residue for peptide a, *P EGD*. Proposed residue for peptide b, *LIVTQTMK*.

TABLE 2. Sequence of amino acids for the fragments of peptides c and d after mass spectrometry.

| HPLC Peak | Ion mass of fragments | Intensity | Possible sequence of amino acids for fragments | MW[1] of Peptide proposed | Maximum error | Amino acid position in β-LG |
|---|---|---|---|---|---|---|
| | | (% of base peak) | | | (%) | |
| c | 1006.9 | 100 | **TQTMKGL DI**[2] | 1007.19 | 0.06 | 4–12 |
| | 2013.6 | 80 | MKGL DIQKVAGTWY SLAM | 2013.42 | 0.04 | 7–24 |
| | | | IQKVAGTWY SLAMAASDIS | 2013.32 | 0.04 | 12–30 |
| | | | **QKVAGTWY SLAMAASDIS L**[2] | 2013.22 | 0.05 | 13–31 |
| | 965.2 | 24 | SLAMAASDIS | 966.09 | 0.19 | 21–30 |
| | 1294.1 | 16 | L DIQKVAGTWY | 1294.49 | 0.06 | 10–20 |
| | 701.75 | 8 | **LDAQSAP**[2] | 701.75 | 0.02 | 32–38 |
| | 1239.1 | 44 | *DKAL KALPMHI*[3] | 1237.55 | 0.29 | 137–147 |
| | 2478.89 | <8 | **ALPMHIRLS FNTPQLEEQC HI**[3] | 2478.89 | 0.07 | 142–162 |
| | 359.2 | 24 | EGD + (potassium) | 359.28 | 0.13 | 51–53 |
| | 1324.1 | 16 | *DYK KYLLFCM*[4] | 1324.64 | 0.12 | 98–107 |
| | 437.1 | 15 | R VY | 437.52 | 0.21 | 40–42 |
| d | 655.2 | 100 | **TDYK K**[2] | 654.74 | 0.15 | 97–101 |
| | | | **CMENS A**[2] | 654.73 | 0.15 | 106–111 |
| | 611.2 | 96 | TDYK K – COO | 610.74 | 0.15 | 97–101 |
| | | | CMENS A – COO | 610.73 | 0.15 | 106–111 |
| | 639.4 | 82 | TDYK K – O | 638.74 | 0.15 | 97–101 |
| | | | CMENS A – O | 638.73 | 0.15 | 106–111 |
| | 595.2 | 80 | TDYK K – CH3COOH | 594.74 | 0.15 | 97–101 |
| | | | CMENS A – CH3COOH | 594.73 | 0.15 | 106–111 |
| | 806.2 | 92 | *LIVTQTM*[3] | 806.01 | 0.05 | 1–7 |

[1]Molecular mass.

[2]Fragments of the main peptide (in bold). Proposed sequence of amino acids for peptide c, **TQTMKGL DIQKVATWY SLAMAASDIS LDAQSAP**. Proposed sequence of amino acids for peptide d, **TDYK K** or **CMENS A**.

[3]Residual peptides (in italics). Proposed residue for peptide c, *DKAL KALPMHIRLS FNTPQLEEQC HI*. Proposed residue for peptide d, *LIVTQTM*.

[4]This sequence contains the bioactive site *YLLF*.

(Table 2). In peak c, residues of *DKAL KALPMHIRLS FNPTQLEEQC HI* and DYK KYLLFCM were also detected, which contain the bioactive sites HIRL YLLF, respectively.

The maximum MW error for the different fragment constituents of peak a was 0.02% (wt/wt) and for peak b was 0.15% (wt/wt) (Table 1). The maximum error for the fragments of peak c was 0.19% (wt/wt) and for peak d was 0.15% (wt/wt) (Table 2). The maximum error for the residues were 0.45% (wt/wt) for peak a, 0.18% for peak b (Table 1), 0.29% for peak c, and 0.05% for peak d (Table 2).

## DISCUSSION

*Kluyveromyces marxianus* var. *marxianus* metabolizes soluble whey proteins during fermentation as indicated by the oscillatory degradation of whey protein during fed-batch fermentation. This profile may occur because of the interaction of two independent phenomena: 1) the consumption of soluble proteins, mainly lactoglobulin, and 2) the release of proteases and peptidases by the yeast during fermentation. The former was responsible for the consumption of proteins mainly during the batch fermentation. The latter was responsible for the accumulation of protein during the fed-batch stage. Proteases and peptidases were thus required by *K. marxianus* to hydrolyze the soluble proteins and to convert them into oligopeptides. Whey proteins were probably used as an alternative source of both N and C by *K. marxianus*, which was confirmed by the consumption of the OP **TQTMKGL DIQKVAGTWY SLAMAASDIS LDAQSAP** (peak c) at the final stages of the fermentation when the lactose concentration was low and the yeast cells were aging. The detection of this OP with a retention time of 37.5 min in all samples collected during fermentation suggests that proteases from *K. marxianus* have specificity to break V-**T** (f 3–4) and **P**-L (f 38–39) bonds of lactoglobulin. The residues found in peaks b and d (corresponding to *QTMK* and *LIVTQTM*) suggest that peptidases from *K. marxianus* have specificity to break **T-Q** (f 4–5), **K-G** (f 8–9) and **M-K** (f 7–8) bonds of the same oligopeptide.

The production and accumulation of **TDYK KYLLFCMENS** or **DYK KYLLFCMENS A** (peak b) during fermentation was detected by HPLC. These peptides contain the $\beta$-lactorphin site YLLF (11), which suggests that whey fermentation may be an alternative process for the production of antihypertensive compounds. However, the area of these peptides significantly increased only after 40 h of fermentation during the fed-batch stage of fermentation, which implies that the production of anti-hypertensive compounds by *K. marxianus* grown on whey should be conducted in fed-batch conditions. However, this peptide is much longer than $\beta$-lactorphin. Therefore, the active site still may be hidden by the peptide configuration, and it may not present any antihypertensive properties.

The accumulation of **TDYK KYLLFCMENS** or **DYK KYLLFCMENS A** (peak b) in the wort also suggests that proteases from *K. marxianus* have specificity to break either D-**T** or T-**D** (f 96–97 or f 97–98) bonds and either **S**-A or **A**-E (f 110–111 or f 111–112) bonds. Residues of DYK KYLLFCM and **TDYK K** or **CMENS A** in peaks c and d suggest that peptidases from *K. marxianus* have specificity to break those OP (f 97–110 or f 98–111) and to separate the $\beta$-lactorphin site YLLF (11). Proteases and peptidases from *K. marxianus* are not able to break the peptidic bonds of YLLF, and this site remains intact during fermentation. Thus, the use of selected proteases and peptidases from this yeast may be examined to produce bioactive peptides through enzymatic procedures. Moreover, the accumulation of **RV Y** (f 40–42), peak a, during whey fermentation suggests that this peptide has potential for further studies to verify its application in food processing.

The low errors verified for the different fragments, all less than 0.45% (wt/wt), indicate the accuracy of the sequence of amino acids proposed for peaks a, b, c, and d by using only MS.

## CONCLUSIONS

Whey fermentation by *Kluyveromyces marxianus* may be a promising and viable process to obtain bioactive peptides derivated from lactoglobulin. Their applications in food products, however, still have to be tested as some peptides are implicated in attributing bitter taste to foods.

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