



Optimised purification and characterisation of lipid transfer protein 1 (LTP1) and its lipid-bound isoform LTP1b from barley malt



Melanie Nieuwoudt^{a,b}, Nicolaas Lombard^a, Marina Rautenbach^{a,*}

^a BIOPEP Peptide Group, Department of Biochemistry, Science Faculty, University of Stellenbosch, South Africa

^b Department of Food Science, Faculty of AgriScience, University of Stellenbosch, South Africa

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ABSTRACT

In beer brewing, brewers worldwide strive to obtain product consistency in terms of flavour, colour and foam. Important proteins contributing to beer foam are lipid transfer proteins (LTPs), in particular LTP1 and its lipid-bound isoform LTP1b, which are known to transport lipids *in vivo* and prevent lipids from destabilising the beer foam. LTP1 and LTP1b were successfully purified using only five purification steps with a high purified protein yield (160 mg LTP1 and LTP1b from 200 g barley). Circular dichroism of LTP1 and LTP1b confirmed that both proteins are highly tolerant to high temperatures (>90 °C) and are pH stable, particularly at a neutral to a more basic pH. Only LTP1 exhibited antiyeast and thermo-stable lytic activity, while LTP1b was inactive, indicating that the fatty acid moiety compromised the antimicrobial activity of LTP1. This lack in antiyeast activity and the positive foam properties of LTP1b would benefit beer fermentation and quality.

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1. Introduction

One of the most important contributing factors towards beer quality is beer foam (Bamforth, 1985). Beer foam consists of interactions and relations between a variety of factors such as hop acids, proteins, polysaccharides, the presence of metal ions, foam destabilising factors, ethanol, yeast protease activity and more (Bamforth, 1985; Evans & Bamforth, 2009).

It is already widely known that protein Z and lipid transfer protein 1 (LTP1) are two of the major contributors to the formation and stability of beer foam (Evans & Bamforth, 2009). Both proteins can withstand and tolerate the high temperatures used during brewing (Lindorff-Larsen & Winther, 2001). LTP1 is a 9 kDa basic protein (Jegou, Douliez, Molle, Boivin, & Marion, 2000) with a pI >9 (Lindorff-Larsen & Winther, 2001). This protein can non-specifically bind a wide range of lipids in its modified form (Stanislava, 2007) and so doing prevent lipids from destabilising beer foam. Modification of LTP1, such as glycosylation, occurs when the protein is exposed to the high temperatures of the brewing process (Jegou et al., 2000). Post-translational modification of LTP1, due to a lipid-like adduct covalently bound to it (Perrocheau, Bakan, Boivin, & Marion, 2006), leads to the formation of LTP1b, which

is 294 Da larger than LTP1 (Mills et al., 2009). This adduct of LTP1b was identified to be α -ketol-9-hydroxy-10-oxo-12(Z)-octadecenoic acid (Bakan et al., 2006) and is formed when linoleic acid is oxygenised to form a hydroperoxide that is subsequently dehydrated (Matejkova et al., 2009).

LTP1 has numerous proposed biological functions of which one is acting as an antimicrobial protein for protecting the barley grain from infections in the field (Lindorff-Larsen, Lerche, Poulsen, Roepstorff, & Winther, 2001). It is theorised that LTP1 acts on the membranes of pathogens and other microbes (Kader, 1996). It is of importance to the brewing industry to fully understand the effect of the presence of these proteins or peptides during processing. LTP1 has been shown to have inhibitory effects on the common lager brewing yeast, *Saccharomyces cerevisiae* (Gorjanovic et al., 2004; Van Nierop, Axcell, Cantrell, & Rautenbach, 2008), as well as some fungal pathogens occurring in the field, which can be detrimental to beer quality.

Due to the LTP1s resistance to high temperatures, it will survive the mashing process and still be present during fermentation (Gorjanovic et al., 2004). The peptides are thus not only important to brewers as a major contributor towards foam formation but will also influence fermentation. Previous research indicated that neither LTP1 nor LTP1b unfolded completely at temperatures reaching 100 °C (Matejkova et al., 2009; Perrocheau et al., 2006) and will thus be necessary to take into account throughout the brewing process.

* Corresponding author. Address: Department of Biochemistry, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa. Tel.: +27 21 8085872; fax: +27 21 8085863.

E-mail address: mra@sun.ac.za (M. Rautenbach).

LTP1s account for 5–10% of all soluble proteins (Stanislava, 2007) in grains and have, in past, been isolated from barley (Douliez et al., 2001; Garcia-Casado, Crespo, Rodriguez, & Salcedo, 2001; Gorjanovic et al., 2004; Gorjanovic et al., 2005; Lindorff-Larsen et al., 2001; Lusk, Goldstein, & Ryder, 1995; Matejkova et al., 2009; Mills et al., 2009; Sorensen, Bech, Muldbjerg, Beenfeldt, & Breddam, 1993), wheat (Tassin-Moindrot, Caille, Douliez, Marion, & Vovelle, 2000) and various other plant materials (Molina, Segura, & Garciaolmedo, 1993; Oshchepkova et al., 2009; Zaman & Abbasi, 2009; Zoccatelli et al., 2007). During this study optimisation of the existing purification methods for barley LTP1 and LTP1b were roughly based on the method described by Sorensen et al. (1993). Purified proteins were characterised with SDS–PAGE, immunological detection and analysis, electrospray mass spectrometry, circular dichroism as well as assessed in terms of its activity towards strains of *S. cerevisiae*.

2. Materials and methods

2.1. Materials

2.1.1. Raw material and chemicals

Barley (*Hordeum vulgare*), cultivar SSG 506 and *S. cerevisiae* SAB5 were supplied by SABMiller (Caledon, South Africa). *S. cerevisiae* NFP was from National Food Products (Emmentaria, Johannesburg, South Africa). Chemicals and other materials were obtained from the following companies: tri-sodium citrate from B&M Scientific cc (Cape Town, South Africa); ammonium sulphate and acetic acid from Kimix (Eppindust, South Africa); Filtraflow 4252 from INFIGRO Natural Technologies (Pty) Ltd (Olifantsfontein, South Africa); Celetom FW-13, Eagle Pitcher Filtration & Minerals Inc. (Reno, Nevada, USA); Sephadex G25 and G50 from Pharmacia Fine Chemicals (Uppsala, Sweden); CM Sepharose from Amersham Pharmacia Biotech AB (Uppsala, Sweden); Tris from Melford Laboratories Ltd (Ipswich, UK); Coomassie blue R-250, SDS sample buffer and low molecular weight markers from Sigma Aldrich (Missouri, USA); nitrocellulose membranes from Pall Corporation (Florida, USA); goat anti-rabbit HRP-conjugate from Bio-Rad (Parklands, South Africa); immunoblotting substrate from Pierce (Thermo Scientific, Rockford, Illinois, USA); propidium iodide were from Sigma–Aldrich (St. Louis, USA). LTP1 generated primary antibodies were generously donated by Prof. Evan Evans (School of Plant Science, University of Tasmania, Australia). Analytical quality water was prepared by filtering water from a reverse osmoses plant through a Millipore Milli Q[®] water purification system (Milford, USA).

2.2. LTP1 purification

The five step purification protocol for LTP1 and lipid associated analogue LTP1b from barley is depicted in Fig. 1 and described in detail hereafter. One hundred grams barley was milled using a Cyclotec 1093 sample mill from Foss Tecator (Hillerod, Denmark) and extracted for 20 h with 300 mL analytical quality water at 4 °C. In the case of isolating unmodified LTP1, barley was defatted first as was recommended by Bakan et al. (2006) by washing the kernels with a 60% ethanol solution and subsequently drying it in a vacuum dryer overnight. For LTP1b isolation the ethanol wash step was omitted. The extracted milled barley slurry was centrifuged at 4 °C for 20 min at 17,600g (10,000 RPM using a Beckman JA-10 rotor).

The supernatant was saturated to 40% ammonium sulphate and filtered using filter aids: 1.0 g Filtraflo 4251/10 cm² filtration surface and 2 g Celetom FW-13 per 100 mL filtrate. The filtrate was further saturated with ammonium sulphate up to 80% and

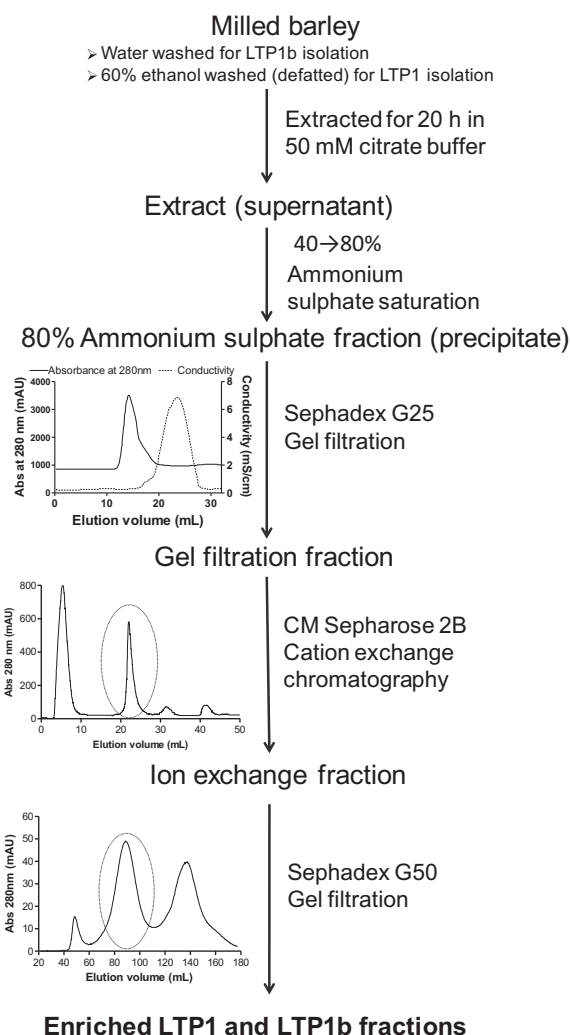


Fig. 1. A Stepwise flow diagram of the LTP1 and LTP1b isolation procedure from barley. Circled elution peaks indicate LTP1 positive fractions.

centrifuged at 17,600g at 4 °C for 20 min. The 80% ammonium sulphate precipitate was suspended in analytical quality water and desalted using a 1.6 × 18 cm Sephadex G25 column connected on an AKTA purifier (Amersham Pharmacia Biotech, Uppsala, Sweden). A 1% acetic acid solution was used to elute the protein fractions at a flow speed of 0.5 mL/minute. The protein containing fractions collected were concentrated by freeze drying.

The LTP1 containing fraction, as determined with SDS–PAGE and dot immune blot, was dissolved in analytical quality water and applied to a CM–Sepharose column (4 mL bed volume) equilibrated with 5 mM Tris–HCl (pH 7.0) and a flow rate of 0.5 mL/minute. Contaminating proteins were separated from LTP1 (or LTP1b) by running a stepwise gradient. After loading the protein fraction, 5 mM Tris–HCl (pH 7.0) was applied for five column volumes, followed by 50 mM Tris–HCl at pH 9.0 for five column volumes and finally a step gradient of 50 mM Tris–HCl, 0.5 M NaCl at pH 9.0 for 3 column volumes.

The LTP1 (or LTP1b) containing fraction, as determined by SDS–PAGE and western blot, was concentrated by freeze drying. The freeze-dried LTP1 fraction was further purified on a 1.5 × 36 cm Sephadex G50 column and eluted with 1% acetic acid at a flow speed of 0.5 mL/min. Protein fractions collected were analysed using SDS–PAGE, electrospray mass spectrometry (ESMS), circular dichroism (CD) and antiyeast activity assays.

2.3. Analysis of protein fractions

2.3.1. SDS-PAGE analysis

Fractions were pre-boiled at 90 °C for 10 min with an equal amount of sample buffer and subsequently subjected to SDS-PAGE. The gels were cast by making use of Hoefer Mighty Small Dual System from Hoefer Inc. (Holliston, USA). A 15% SDS-polyacrylamide stacking gel and 5% separating gel was used to analyse fractions as described by Laemmli (Laemmli, 1970). Ultra low range molecular weight markers were used as size indicators. The gel was run under a constant current of 20 mA. The gel was removed and stained for 1 h using the staining solution, 0.125% Coomassie blue R-250, 50% methanol and 10% acetic acid. Thereafter it was destained with destain solution 1, 50% methanol and 10% acetic acid for another hour and lastly placed in destain solution 2, 7% acetic acid and 5% methanol until the gel was completely clear. The gel was subsequently digitised using UN-SCAN-IT gel 1.6 software package (Silk Scientific Inc, Orem, Utah).

2.3.2. Dot and western blot analyses

Immunological assays were performed using anti-LTP1 polyclonal antibodies. For the dot immuno-blot, 2 µL of each fraction was placed on a strip of nitrocellulose membrane. The membrane was incubated with casein buffer (154 mM NaCl, 0.5 casein, 10 mM Tris-HCl and 0.02% thiomersal, pH 7.2) for 20 min while slowly shaken. This was followed by the incubation of the membrane with whole serum (1:5000 v/v) and further incubated for 60 min at room temperature. The membrane was then washed four times for 5 min with PBS-Tween (0.8% NaCl, 0.02% KCl, 0.115% Na₂HPO₄, 0.02% KH₂PO₄, 0.1% Tween 20, pH 7.2) after which it was placed in casein buffer with goat anti-rabbit horse radish peroxidase-conjugate for another 60 min at room temperature. The membrane was then washed again four times for 5 min with PBS-Tween and incubated for 1 min with Pierce ECL western blotting substrate after which it was exposed and developed.

A western blot was performed using the same antibodies as for the dot immuno-blot. The proteins separated on the SDS-PAGE gel were transferred onto a nitrocellulose membrane at 0.8 mA/cm² gel. The membrane with the transferred proteins was incubated with 20 mL casein buffer (154 mM NaCl, 0.5 casein, 10 mM Tris-HCl and 0.02% thiomersal, pH 7.2) for 20 min under slight agitation. This was followed by the incubation of the membrane with whole serum (1:5000 v/v) and further incubation for 60 min at room temperature. The membrane was then washed four times for 5 min with 20 mL PBS-Tween (0.8% NaCl, 0.02% KCl, 0.115% Na₂HPO₄, 0.02% KH₂PO₄, 0.1% Tween 20, pH 7.2) after which it was placed in 20 mL casein buffer with goat anti-rabbit HRP-conjugate (1:10,000 dilution) for another 60 min at room temperature. The membrane was then washed again four times for 5 min with 20 mL PBS-Tween and incubated for 1 min with Pierce ECL western blotting substrate after which it was exposed 15 min and developed.

2.3.3. Mass spectrometry

Samples were prepared by diluting the purified LTP protein or enriched extract in analytical quality water to yield a final concentration of 200–1000 µg/mL. Electrospray mass spectrometry (ESMS) was performed using a Waters Quadrupole Time-of-Flight Synapt G2 mass spectrometer. A Waters Acquity UPLC injector was used to inject 2 µL of each sample into a Z-spray electrospray ionisation source in positive mode to analyse direct mass. A capillary voltage of 3.0 kV and cone voltage of 15 V were applied at a source temperature of 120 °C. Data acquisition was in the positive mode with scanning from *m/z* 150 to 2000. Molecular masses from the multi-protonated spectra's were calculated over 2000–40,000 Da mass range with 20 charges set as maximum and

automatic peak width detection using MaxEnt 3 algorithm from MassLynx V4.1.

2.4. Characterisation

2.4.1. Heat and pH stability

LTP1/1b fractions (0.200 mg/mL) were analytically prepared in analytical quality water for temperature stability determination and in 40 mM phosphate-buffered saline adjusted with either 6 M NaOH or 6 M HCl, to the desired pH, for the pH stability experiments. The pH range buffers at which LTP1 and LTP1b enriched preparations were tested, included pH 1.0, 3.0, 5.0, 7.0, 9.0, and 11.0. Solvents were individually scanned for each respective pH and subtracted as baseline from each of their respective samples.

The temperature and pH stability of LTP1 and LTP1b preparations were obtained on a Chirascan Plus CD spectrometer (Applied Photophysics, UK). All analyses were conducted at room temperature using a quartz cell with a path length of 0.5 mm. CD scans in triplicate were from 190 to 260 nm at a band width of 1 nm and data collection of 0.5 s per nm. For the temperature related experiments, a metal probe was inserted into the sample cell to record the sample temperature. During the temperature ramping, each scan was repeated five times and data were accumulated at each nm was for 0.2 s at the varying temperatures. Scans were performed, starting at 25 °C and with 5 °C increments until a temperature of 95 °C was reached. The solvent used was scanned and subtracted as baseline from each respective temperature spectra.

2.4.2. Antiyeast activity

A dose response was performed for each of the peptide enrichments towards two different *S. cerevisiae* strains (SAB5 and NFP), as previously described by Van Nierop, Axcell, Cantrell and Rautenbach (2008). The yeast strains were both grown in MYGP broth (3% each of malt and yeast extract, 5% peptone, 10% glucose) at 30 °C from OD 0.01 to 0.5 at 595 nm and diluted 200 fold before inoculation. Doubling dilutions were made with analytical quality water of both peptide enrichments starting at 800 µg/mL and decreasing to 12.5 µg/mL. Gramicidin S was used as a positive control and was also applied in a doubling dilution series starting at 8 µg/mL, decreasing to 0.125 µg/mL. For each peptide, the assay was done in duplicate with triplicate technical repeats per assay. Plates were incubated at 30 °C for 16 h and yeast growth was determined at 595 nm in a microtiter plate reader after being shaken on medium speed for 5 s. The results were analysed as described by Rautenbach, Gerstner, Vlok, Kulenkampff, and Westerhoff (2006).

2.4.3. Lytic activity

To determine whether the high temperatures of the brewing process would result in a loss of antiyeast activity the purified LTP1 was boiled for 5 and 10 min at 100 °C and evaluated by a membrane permeability assay as previously described by Bink et al. (2012) using a membrane impermeable dye propidium iodide. Yeast was prepared in a similar manner for the membrane permeability assay as for the dose response assays. LTP1 was used in concentrations five and ten times greater than its IC₅₀ (75 and 150 µM) and subjected to the various heat treatments. Assays were performed in 96 well black microtiter plates used for fluorescence measurements. All samples, growth controls and blanks were treated with 1% propidium iodide. Gramicidin S (17.5 µM) and 1% Triton X-100 served as lytic controls. The propidium iodide emitted fluorescence was recorded using a Varioskan (Thermo Scientific, Waltham, MA, USA).

3. Results and discussion

3.1. Purification

The optimised five step purification protocol for LTP1 and LTP1b is depicted in Fig. 1 and discussed in detail below. To optimise the existing purification methods for barley LTP1 and LTP1b, with emphasis on the method described by Sorensen et al. (1993), we opted to generally handle LTP similar to peptides, as this is a very stable small protein that survives the brewing process (Evans & Bamforth, 2009; Van Nierop, Evans, Axcell, Cantrell, & Rautenbach, 2004). First, an extract was made from barley flour by precipitating it at 40%, followed by an 80% ammonium sulphate in the second step. In the third step the dissolved ammonium sulphate precipitate was desalted on a Sephadex G25 column and this method of desalting proved to be highly efficient as very little protein was lost during this step. We opted for size-exclusion (gel-permeation) chromatography for both desalting and purification, rather than dialysis or ultra-filtration. In our experience amphipathic peptides and proteins such as the LTPs tend to associate with ultra-filtration and dialysis membranes leading to major losses. As LTP1 is very stable, similar to many disulphide bonded antimicrobial peptides, we exchanged the high salt buffers in size-exclusion chromatography steps (steps 3 and 5) with 1% acetic acid. This helped to remove acid labile proteins and eliminate the buffer exchange step, as well as easing the freeze drying of purified fractions.

The desalted protein fraction was further purified *via* cation exchange chromatography on a carboxymethyl-modified resin in the fourth step. The pH 7 buffer was used to elute all proteins with $pI \leq 7$ from the column ("void" in Fig. 2A). Since LTP1 has a pI of 8.19, it was expected to elute with a buffer at $pH > 8$, but two protein fractions (fractions 1 and 2) eluted at pH 9 and one fraction at pH 9 with high [NaCl] (Fig. 2A). Fractions 1 and 2, evaluated using SDS-PAGE (results not shown) and dot immuno-blotting using a polyclonal anti-LTP1 antibody preparation, contained LTP1 like proteins (Fig. 2A). It is clear from Fig. 2 that fraction 1 contained most of the proteins reactive to the LTP1 antibody, although fraction 2 also had a similar sized protein at 9–10 kDa, but with less prominent reactivity towards the anti-LTP1 antibodies.

To further purify the LTP containing fraction 1, the fifth and final purification step entailed another size exclusion chromatography step (Fig. 2B). Three fractions were found (Fig. 2B) which correlated with the SDS-PAGE results (not shown) of fraction 1 indicating a mixture of different sized proteins apart from the LTP1. ESMS of peak fraction 2 confirmed that the fraction contained about 70% pure LTP1 or LTP1b, depending if ethanol wash was included or not.

Bakan et al. (2006) found that barley malt washed with a 60% ethanol solution yielded more LTP1 while unwashed barley would yield a higher amount of LTP1b. This washing principle (defatting) was therefore applied as the first step. The purified protein fraction from the defatted barley predominantly consisted of the unmodified LTP1 isoform with an experimentally determined M_r of 9680.85 (expected 9680.53) and minor contributions of LTP1b and other isoforms (Fig. 3A). These M_r -values for LTP1 and LTP1b compared well with those determined by (Jegou, Douliez, Molle, Boivin, & Marion, 2001; Jegou et al., 2000) and Van Nierop et al. (2004) using ESMS. A small amount of a protein with an M_r of 9517.82 was also detected, which could be LTP1b without its C-terminal tyrosine residue (see insert Fig. 3A). When the purification protocol was repeated using barley malt not defatted by the ethanol wash we were able to purify LTP1b as major component. ESMS revealed that the amounts of the two major isoforms reversed, yielding more of the LTP1b isoform with experimentally determined M_r of 9973.75 (expected 9974.74) and less LTP1

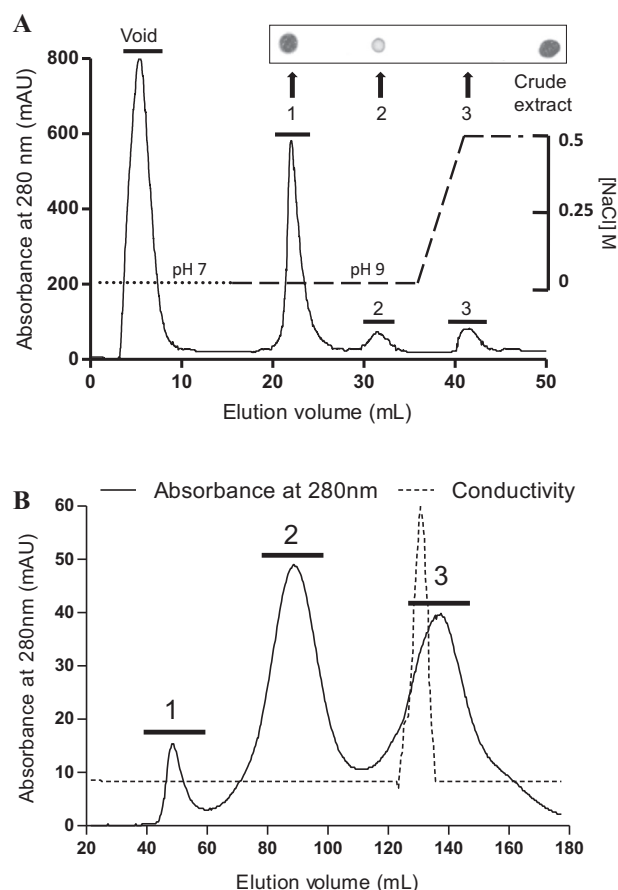


Fig. 2. (A) A representative cation exchange chromatogram obtained from a CM Sepharose column separation of the desalted 80% ammonium sulphate fraction as developed with a stepwise gradient of 5 mM Tris-HCl (pH 7), 50 mM Tris-HCl (pH 9) and 50 mM Tris-HCl + 0.5 M NaCl (pH 9). The bold lines above the peaks indicate the pooled peak fractions. Dot immuno-blot analysis, using anti-LTP1 antibodies of the three different fractions (1–3) and crude extract, indicated positive dots correlating to LTP1. (B) A representative Sephadex G50 size exclusion chromatogram of the final purification step of the LTP1 containing fraction 1 from cation-exchange chromatography step depicted in A, using 1% acetic acid as eluant. The bold lines above the peaks indicate the pooled fractions.

(Fig. 3B). A putative dimer of LTP1b was also detected at $M_r = 19356.66$, as well as hydroxylated forms at 9991.75 and 10007.15 (Fig. 3B).

A total yield of 880 mg/kg and 720 mg/kg was obtained from barley malt for LTP1 and LTP1b respectively (refer [Supplementary data Table 1](#) for more detail on purification yields). Reported LTP1 yields from literature were 200 mg LTP1/kg and 100 mg LTP1b/kg barley malt (Douliez et al., 2001), 103.3 mg/kg LTP1 and 82.7 mg/kg LTP1b (Jegou et al., 2000) and 187.5 mg LTP1b/kg barley malt (Mills et al., 2009). These reported methodologies had more than three chromatography steps, with some incorporating dialysis and/or ultra-filtration steps, all of which can lead to cumulative losses. Our high yields were obtained because losses were limited using only five purification steps and by elimination of dialysis and ultra-filtration steps. Furthermore, ESMS analysis for the two protein preparations confirmed that LTP1 and LTP1b retained their four native disulphide bonds and that all the peptide bonds (primary structure) were intact, even though rather harsh isolation procedures were used (Fig. 3). The robust and optimised five-step LTP1 and LTP1b purification protocol can also be easily up-scaled for commercial or industrial purification of LTP1.

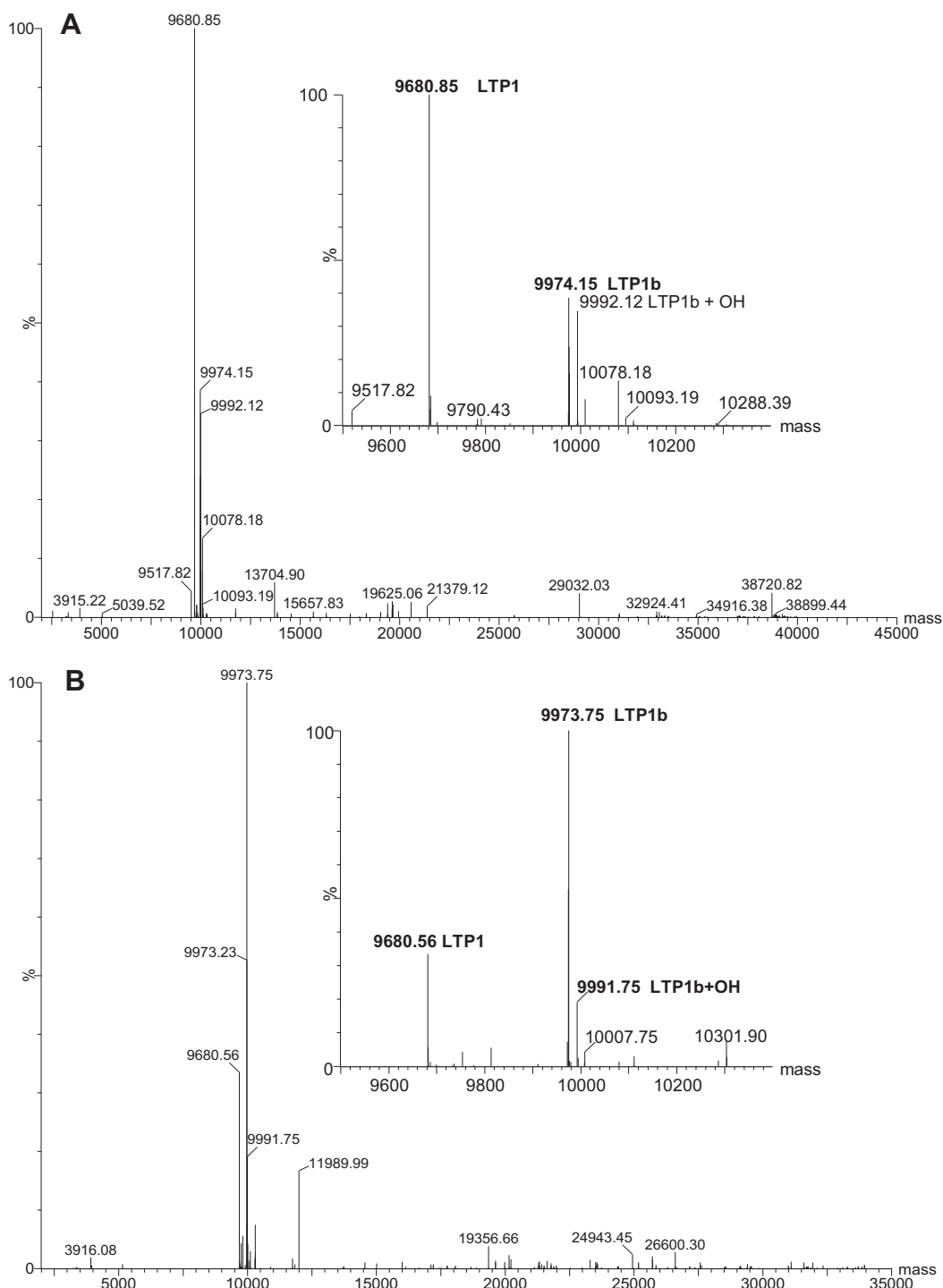


Fig. 3. Positive mode ES-MS mass generated* spectra of the Sephadex G50 purified fraction 2 from (A) defatted malt extract and (B) from malt which was not defatted. The LTP1 isoform is indicated at 9680.85 Da (major peak) and the LTP1b isoform is indicated at 9974.15 Da in A and the LTP1b isoform is indicated 9973.74 Da (major peak) and the LTP1 isoform is indicated at 9680.85 Da in B. *Mass spectra were generated with the MaxEnt 3 algorithm from MassLynx V4.1. The inserts in each of the two larger spectra show the 9500–10,500 Da region.

3.2. Temperature and pH stability as determined by circular dichroism

To probe the structure and better understand the thermal changes undergone by LTP1 and LTP1b during the brewing process, both peptides were evaluated over a wide temperature range using circular dichroism (CD). Due to LTP1's major contribution towards beer foam, thermal studies on its secondary structure have been

performed, also by making use of CD by a number of investigators (Jegou et al., 2000, 2001; Pato, Tran, Marion & Douliez, 2002; Mills et al., 2009; Perrocheau et al., 2006; Van Nierop et al., 2004). Although we analysed mixtures of LTP1 and LTP1b, each preparation were highly enriched and the dominant protein would have the largest influence on the spectra. The spectra of both the preparations showed a typical CD spectrum of a protein containing

predominantly α -helices, as found in LTP1. From the spectra (Fig. 4) two minima's are observed at 208 and 222 nm respectively for both isoforms indicating the presence of α -helices known to be an integrate part of the secondary structure of LTP1 and LTP1b (Jegou et al., 2000; Mills et al., 2009). Similar minima's were observed by Van Nierop et al. (2004; Mills et al. (2009)) and Jegou et al. (2001). LTP1 showed a much more pronounced 208 nm minimum with 208 nm/222 nm ratio of 1.20, while LTP1b had a 1.15 ratio, indicating the isoforms have secondary structure differences, possibly induced by the fatty acyl modification of LTP1b.

Upon heating most of the changes in ellipticity for each LTP1 isoform took place, as expected for a protein, at the far-UV CD region between 200 and 260 nm. A decrease of ellipticity and slight shift in the two minima's was observed upon heating, which indicated that both proteins started to partially unfold, but loose only 15–33% of their secondary structure. The spectra of LTP1b seemed

to change more over the temperature range with an overall change of 24% at 208 nm and 33% at 222 nm from 25 °C to 90 °C, while there was only a 15% and 20% change for LTP1, respectively. This is comparable to the 25% loss of structure observed by Mills et al. (2009) for both LTP1 and LTP1b and the 10–15% loss for LTP1 structure as found by Van Nierop et al. (2004). However, when samples were cooled down to the original starting temperatures of 25 °C CD spectra of both LTP1 and LTP1b indicated that they are able to fold back close to their original state, with the refolded protein spectra differing less than 10% and 16% from the original spectra for LTP1 and LTP1b, respectively.

When the spectra of the LTP1 isoform preparations were recorded under different pH conditions, both isoforms, again, showed two minima's at 208 and 222 nm characteristic of α -helical structures. Only a 15–33% decrease in ellipticity was observed for both LTP1 and LTP1b as the pH moved away from neutral

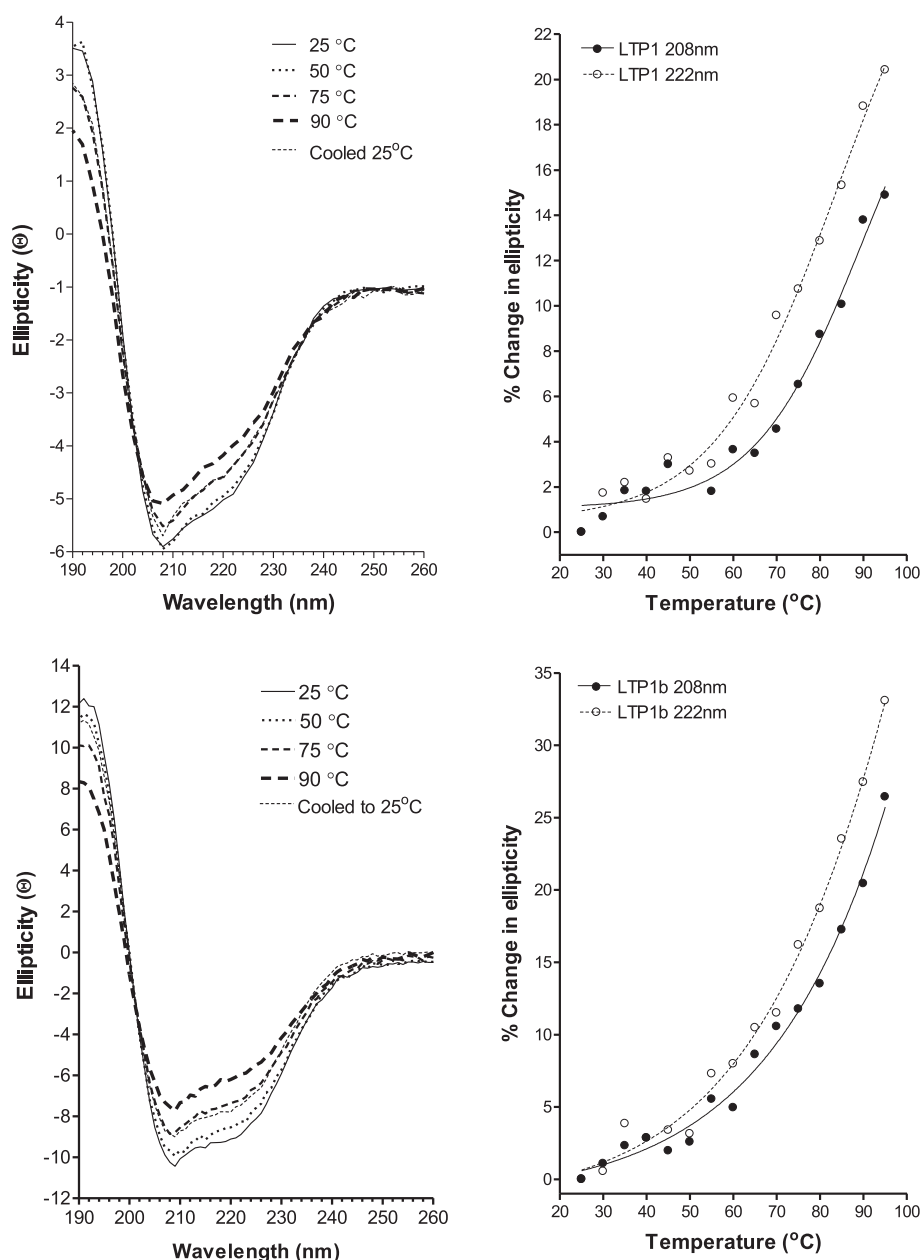


Fig. 4. CD spectra for LTP1 (top left) and LTP1b (bottom left) over a temperature range of 25 °C to 90 °C. Percentage change in ellipticity of the α -helical structures found in LTP1 (top right) and LTP1b (bottom right) at 208 and 222 nm on the CD spectra over temperature increase from 25 to 95 °C.

(Fig. 5; top right for LTP1 and bottom right for LTP1b). The spectra of LTP1b seemed to change more from pH 7 to pH 1 with an overall change of 26% at 208 nm and 33% at 222 nm, while there was only a 15% and 20% change for LTP1 respectively. However, when pH was increased from pH 5 to pH 11, LTP1 and LTP1b CD spectra's indicated that both are able to remain close to their original state, with the states differing less than 22% and 10% from the structure for LTP1 and LTP1b at neutral pH, respectively. It seems if the LTP isoforms are most stable in neutral environments, but still relatively stable in basic environments.

3.3. Antiyeast activity

Gorjanovic et al. (2004) illustrated that LTP1 possesses anti-yeast activity and therefore the maintenance of the expected LTP1 antimicrobial activity is a good indicator of three-dimensional structural integrity and identity. LTP1 and LTP1b were

evaluated for their ability to inhibit the growth of two different strains of *S. cerevisiae*. The strains chosen were a commercially available brewing strain, further on referred to as NFP and a brewing yeast currently being used in the brewing industry, referred to as SAB5. From observation SAB5 grew slightly slower than NFP, but it seemed to be inhibited slightly less by LTP1 than the NFP strain (Table 1). The activity of the LTP1 were in the low micromolar range (15–25 μ M), only 3- to 4-fold less active than gramicidin S, a highly antifungal peptide (Troskie, Vlok & Rautenbach, 2012), indicating a potent antiyeast activity and retention of this activity in our purified fraction.

We observed, using light microscopy, that LTP1 probably interacts with its yeast target membrane leading to lyses within a few minutes (results not shown) correlating with previous observations by Gorjanovic et al. (2004) and Cvetković, Blagojević, Hranisavljević and Vučelić (1997). This activity is most likely due to the positively charged LTP surface binding to the negatively

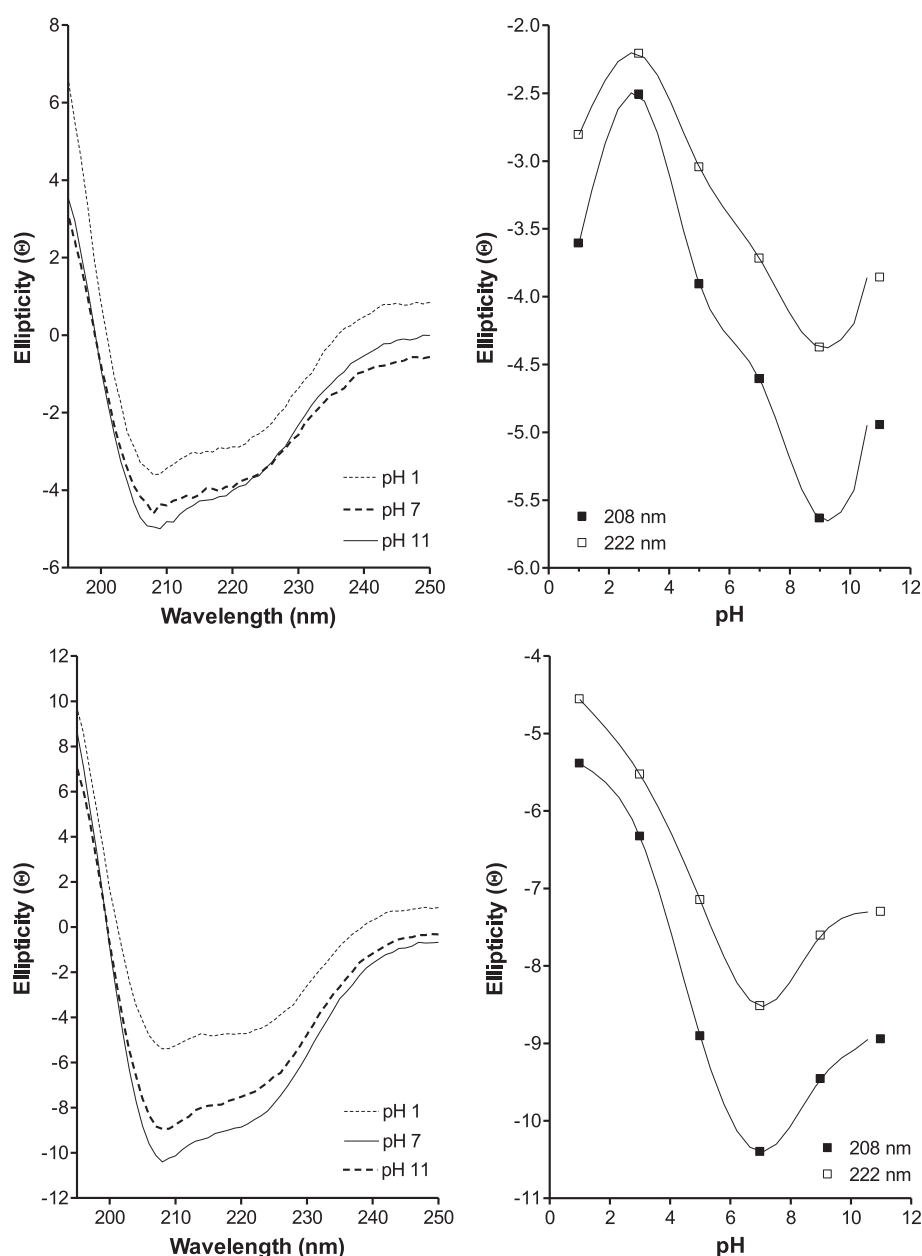


Fig. 5. CD spectra for LTP1 (top left) and LTP1b (bottom left) at pH of 1, 7 and 11. The left hand graphs show the relationship between ellipticity at 208 and 222 nm on the CD spectra of the LTP1 and LTP1b preparations over the pH range of 1–11.

Table 1

Summary of activity data for LTP1 and LTP1b from barley and gramicidin S, used as control lytic peptide.

Compound	IC ₅₀ , Antiyeast activity against SAB5 strain	IC ₅₀ , Antiyeast activity against NFP strain	%Lytic activity ito TritonX100 ([compound])
LTP1	23 ± 1.2 µM ^a	15 ± 1.3 µM ^a	117 ± 11% (75 µM) ^a
LTP1 heated for 10 min at 100 °C	nd	nd	97 ± 16% (75 µM) ^a
LTP1b	>80 µM ^a	>80 µM ^a	nd
Gramicidin S	5.8 ± 1.0 µM	5.2 ± 0.5 µM	95 ± 20% (17.5 µM)

^a Concentrations calculated from the assumption that the preparations contained >90% LTP isoforms. The values are the average of duplicate experiments with at least triplicate technical repeats ± the standard error of the mean.

charged yeast cell surface (Stanislava, 2007). To determine whether the high temperatures of the brewing process would impact LTP1s antiyeast activity, a membrane permeability assay was performed as described by Bink et al. (2012) using the membrane impermeable dye propidium iodide (PI) which forms fluorescent complexes with nucleotides when it enters damaged cells. The lytic activity of LTP1, as detected via PI leakage into treated yeast cells, was maintained after heating to 100 °C for 10 min and subsequently cooled (Table 1). This correlated well with our observations of the LTP1 temperature stability using CD.

LTP is found in beer in concentrations of <35 µg/mL (Evans & Bamforth, 2009; Van Nierop et al., 2004), depending on the severity of the boil, therefore if the LTP1 antiyeast activity is maintained it may be problematic in brewing as this could lead to yeast inhibition and stuck fermentations. However, Gorjanovic et al. (2004) illustrated that LTP1 does not exhibit its inhibitory effect on fermentation in a brewing setup. Conversely, Van Nierop, Rautenbach, Axcell and Cantrell (2006), suggested that even after the mashing process some of the antiyeast activity of LTP1 stays intact. Our results also indicated that LTP1 maintains its activity after boiling (Table 1). However, it was found that most of the LTP1 was converted into LTP1b during the 20 h malt extraction. Similarly, ESMS analysis of a wort extract prepared using the ammonium sulphate precipitation and desalting step of our optimised protocol, showed that it contained a large proportion of LTP1b, as well as LTP1 and glycosylated LTP1 and LTP1b (refer to supplementary data Fig. 1), with latter correlating to the observations by Jegou et al. (2000). LTP1b was unable to inhibit the growth of any of the two yeast strains up to 80 µM (Table 1), while at 80 µM LTP1 no live NFP yeast cells were present. This might be due to the fact that since LTP1b is already covalently bound to a lipid-like adduct it would not attach itself to the yeast cell membrane to cause cell lyses.

4. Conclusions

The LTP1 and LTP1b isoforms were successfully purified using only five purification steps with a high purified protein yield. The CD characterisation of LTP1 and LTP1b showed slight differences, but both proteins are extremely tolerant to the high temperatures involved in the brewing process. At temperatures reaching >90 °C LTP1 and LTP1b still remained relatively intact and refolds to almost its original state upon cooling back to 25 °C. pH stability studies indicated that these proteins are more stable at neutral to a more basic pH.

LTP1 exhibited antiyeast activity depending on lysis indicating membrane activity, while this activity was lost in LTP1b, indicating that the fatty acid moiety compromised the antimicrobial activity of LTP1. However, it was found that most of the LTP1 was converted into LTP1b during extraction and that this structural change could lead to a loss in activity, as seems to be the case for LTP1b. Furthermore an ESMS analysis of a wort extract indicated that this is probably the case in a brewery setting. LTP1 exhibited antiyeast

activity, while this activity was lost in LTP1b, indicating that the fatty acid moiety compromised the antimicrobial activity of LTP1. Gorjanovic et al. (2004) observed that LTP1 does not exhibit its inhibitory effect on fermentation in a brewing setup. They theorised that the presence of wort components antagonise LTPs inhibitory effect and/or that glycation of LTP during mashing leads to inactivation. Our study further indicated that the formation of LTP1b and its glycosylated isoforms may also account for the lack of overt antiyeast activity in the brewery setting.

The information provided by this study shed light on brewing related issues, in particular problems involving beer foam and problematic fermentations. The lack of this protein could result in poor foam stability, while a high concentration of unmodified LTP1 could negatively influence the fermentation process.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2014.02.076>.

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Supplementary data

Table 1 Summary purification data for LTP1 and LTP1b from barley.

Fraction from protein purification step	Enriched fractions containing	
	LTP1	LTP1b
Fraction 1: Gel filtration with Sephadex G25 ¹	776 mg	688 mg
Fraction 1: Cation-exchange chromatography ²	593 mg	413 mg
Fraction 2: Gel filtration with Sephadex G50 ³	88 mg	72 mg
Experimental $M_r^{*\$}$	9680.85	9973.75
Theoretical M_r^*	9680.53	9974.74

*Monoisotopic mass; ^{\$}Experimental M_r determined with MaxEnt 3 algorithm from MassLynx V4.1.

¹. Amount of salt-free protein extract from 80% ammonium sulphate step.

². Amount of salt-containing protein fraction 1.

³. Amount of salt-free purified LTP1 and LTP1b enriched fractions.

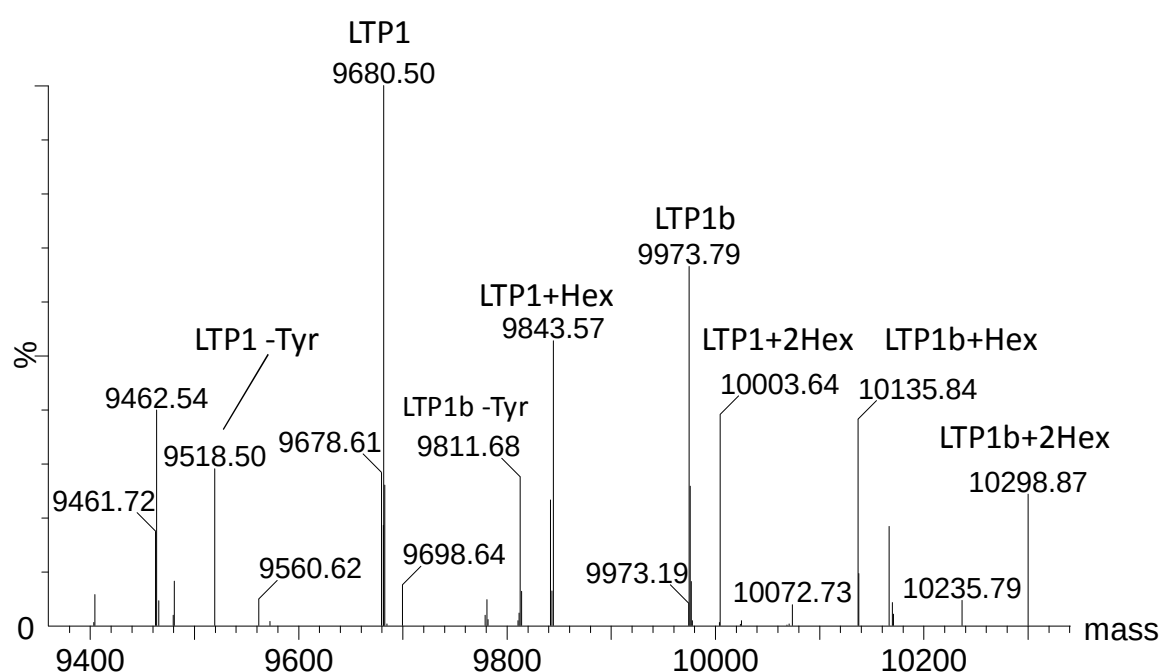


Fig. 1. Positive mode ES-MS mass generated* spectra of a desalted LTP-fraction from a wort extract. The denotation “Hex” indicates that a putative hexose unit was added to the protein structure to form the glycosylated LTP isoform. *Mass spectra were generated with the MaxEnt 3 algorithm from MassLynx V4.1.