

Isolation of activity and partial characterization of large non-proteinaceous lytic allelochemicals produced by the marine dinoflagellate *Alexandrium tamarense*

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Abstract

Certain strains of the toxigenic dinoflagellate *Alexandrium tamarense* produce potent allelochemicals with lytic activity against a wide variety of marine microorganisms. Our efforts to characterize these allelochemicals from a lytic strain focused on the less polar components because of their higher lytic activity. Fractionation and partial purification after solid phase extraction (SPE) were achieved via alternative chromatographic methods, namely HPLC separation on C8 and HILIC phases. Through MALDI-TOF mass spectrometry we compared the mass differences in SPE, C8 HPLC, and HILIC fractions between a lytic and non-lytic strain of *A. tamarense*. Several large species with masses between 7 kDa and 15 kDa were found in the HILIC lytic fraction by MALDI-TOF MS. Tryptic digestion and tryptic digestion-coupled size-exclusion chromatography (SEC) suggested that the lytic compounds are large non-proteinaceous molecules (<23.3 kDa, trypsin). Although there is no direct proof that the large molecules found in the lytic HILIC fraction are responsible for the lytic activity of this fraction, the mass range deduced from SEC strongly supports this hypothesis. Total sugar content analysis showed that the lytic HILIC fraction contained two-fold more sugar than the non-lytic one. Nevertheless, the low percentage of saccharide per dry mass equivalent (0.18 ± 0.01 %) indicates that sugar residues are likely not a major component of the lytic compounds. We concluded that at least one group of lytic allelochemicals produced by *A. tamarense* comprise a suite of large non-proteinaceous and probably non-polysaccharide compounds between 7 kDa and 15 kDa.

Keywords: *Alexandrium tamarense*; allelochemicals; mass spectrometry; toxic dinoflagellate, cell lysis.

1. Introduction

Allelopathy is a biological phenomenon by which an organism produces one or more biochemicals that influence the growth, survival, and/or reproduction of other

co-existing organisms (Rice, 1984). Generation and release of allelochemicals therefore constitutes an evolutionary strategy whereby producers overcome competitors for limited resources, such as space, nutrients, light, etc., or which can serve as a defence mechanism. In marine ecosystems, allelochemicals produced by microalgae may act to deter predators such as heterotrophic protists, tintinnid ciliates and copepods, or to inhibit the growth of co-occurring species (reviewed by Cembella, 2003; Legrand et al., 2003).

In spite of the paucity of knowledge on the structure and function of allelochemicals, allelopathy has long been believed to play a crucial role in phytoplankton bloom formation and succession in both freshwater and marine ecosystems (Pratt, 1966; Keating, 1977; Rice, 1984; Lewis Jr., 1986; Wolfe, 2000; Rengefors and Legrand, 2001; Vardi et al., 2002; Tillmann and John 2002; Legrand et al., 2003; Fistarol et al., 2004; Suikkanen et al., 2005). In recent years, special attention has been paid to allelopathic interactions involving species responsible for harmful algal blooms (HABs) (Granéli and Hansen 2006, Tillmann et al. 2008b). This is partly a reflection of the notorious consequences of HAB events on human activities, such as public health, fisheries, aquaculture and tourism, as well as the devastating effects on aquatic ecosystems. Moreover, the potent toxicity of many HAB species, even at rather low cell concentrations, led to the hypothesis that the production of phycotoxins evidenced an allelochemical mechanism. Structurally well characterized phycotoxins with high potency in mammalian systems, e.g. as neurotoxins or protein phosphatase inhibitors, were suspected to also act as allelochemicals in aquatic ecosystems, thereby spawning much further research on these compounds. For example, diarrhetic shellfish poisoning (DSP) toxins, okadaic acid (OA) and dinophysistoxin-1 (DTX-1), were reported to effectively inhibit the growth of several microalgae (Windust et al., 1996; Windust et al., 1997). However, a later study (Sugg and VanDolah, 1999) found that other compounds must be involved in the toxic effect of the filtrate from the dinoflagellate *Prorocentrum lima*, an OA

producer. Brevetoxins produced by the fish-killing dinoflagellate *Karenia brevis* are not apparently responsible for the allelopathic effects against other phytoplankton species, although they slightly inhibit the growth of the diatom *Skeletonema costatum* (Kubanek et al., 2005). Recent research suggested that *K. brevis* produces multiple allelopathic compounds other than brevetoxins that are inhibitory towards the diatom *Asterionellopsis glacialis* (Prince et al., 2010).

Most allelochemicals from HAB species remain unknown, only a few have been characterized by structure and/or biological activity (Granéli et al., 2008). One allelopathic effect is the direct lysis of target species membranes, thus toxins with hemolytic and/or ichthyotoxic capacity were suspected to have an allelopathic effect. The polyoxy-polyene-polyether toxins prymnesin 1 and 2 produced by the prymnesiophyte *Prymnesium parvum* (Igarashi et al., 1998) are believed to perforate cell membranes of other cells, and can even cause fish kills. Similarly, glycosylglycerolipids with ichthyotoxic and hemolytic potential were found in the fish-killing prymnesiophyte *Chrysochromulina polylepis* and the dinoflagellate *Karenia mikimotoi* (Yasumoto et al., 1990). Karlotoxins (KmTx) with hemolytic activity from the dinoflagellate *Karlodinium veneficum* inhibit the growth of several phytoplankton species (Adolf et al., 2006), and the dinoflagellate grazer *Oxyrrhis marina* (Adolf et al., 2007). The allelochemicals of the raphidophyte *H. akashiwo* that inhibit its diatom competitors *Skeletonema costatum* and *Thalassiosira rotula* were recently identified as high-molecular weight polysaccharide-protein complexes (Yamasaki et al., 2009).

Allelopathy is widely found among *Alexandrium* spp. upon other microalgae (Blanco and Campos, 1988; Arzul et al., 1999; Fistarol et al., 2004; Tillmann et al., 2007; Tillmann et al., 2008) and towards heterotrophic protists (Hansen, 1989; Hansen et al., 1992; Matsuoka et al., 2000; Tillmann and John, 2002). Details of the molecular structures and exact mode of action of allelochemicals from *Alexandrium* species remain scarce. Previous investigation of the major allelochemicals produced

by *A. tamarensis* (Ma et al., 2009) indicated that they are large amphipathic compounds with secondary structure, and are clearly unrelated to the known toxins produced by this genus, namely PSP toxins (Tillmann and John, 2002) or spirolides (Tillmann et al., 2007). Here we further characterized these allelopathic compounds produced by *A. tamarensis* by advanced mass spectrometric techniques. For the identification of candidate masses of compounds related to lytic activity, we compared purified fractions of a lytic and non-lytic strain of *A. tamarensis* by alternative chromatographic and mass spectrometric techniques.

2. Materials and Methods

2.1 Cell culture

One clonal isolate of the marine dinoflagellate *Alexandrium tamarensis* (Alex2) was selected as the source of lytic compounds based on its high lytic activity as quantified comparatively in previous experiments (Alpermann et al., 2009; Tillmann et al., 2009). In some experiments, another isolate Alex5, which does not produce lytic compounds in measurable amounts (Tillmann et al., 2009) and in comparison to Alex2 did not show any allelopathic effects on other algae (Tillmann and Hansen 2009), was used as a negative control. These clones were selected from a collection of >60 clones of the North American ribotype (Lilly et al., 2007) isolated simultaneously in May, 2004 from the Scottish east coast of the North Sea (56° 05' 47'' N; 1° 42' 35'' W). Dinoflagellate cultures were grown in K-medium (Keller et al., 1987), supplemented with selenite (Dahl et al., 1989) prepared from sterile-filtered (VacuCap 0.2 µm Pall Life Sciences) North Sea seawater (salinity 32 psu) in 1 L Erlenmeyer flasks. Cultures were maintained under controlled conditions at 15 °C under cool-white fluorescent light at a photon flux density (PFD) of 100 µmol photons m⁻² s⁻¹ on a 16 h light: 8 h dark photoperiod.

The cryptophyte *Rhodomonas salina* (Kalmar Culture collection; KAC30) cultured under the same condition as *A. tamarensis* described above served as target species to monitor lytic activity at each isolation step, and throughout the various

treatments. The bioassay was performed as described before (Tillmann et al., 2008; Ma et al., 2009).

2.2 Isolation and purification methods

2.2.1 Reversed phase high performance liquid chromatography (HPLC)

Reversed phase solid phase extraction (SPE) fractions were prepared as previously described (Ma et al., 2009). Approximately one liter *A. tamarensis* supernatant, acquired through 15 min centrifugation of cell culture at 3220 x g at 15 °C, was passed over a preconditioned C-18 SPE cartridge (500 mg, 6 mL, Sigma-Aldrich, Deisenhofen, Germany). The cartridge was washed with 10 mL deionized water and 10 mL 20% methanol and eluted with 30 mL 50% methanol and finally 30 mL 80% methanol. The 80% methanol fraction was brought to dryness by rotary evaporation, and the residue was re-suspended in 3.5 mL deionized water, and stored at -20 °C before further use. The concentrated lytic SPE fraction was thawed, and spin-filtered (0.45 µm, Durapore, Millipore) in a centrifuge (Eppendorf 5415R) for 2 min at 15,000 × g at room temperature.

The HPLC separation procedure on a C8 analytical column was performed as previously described (Ma et al., 2009). Ten runs (100 µL injection volume) of fractions with retention of 18 to 19 min were pooled and dried under N₂, and frozen at -20 °C before further use.

2.2.2 Hydrophilic interaction ion-chromatography (HILIC)

Fifty µL 80% methanol SPE fraction, purified from approximately 600 mL supernatant, was separated on an analytical column (150 × 4.6 mm) packed with 5 µm ZIC-HILIC, 200 Å particles (SeQuant, Haltern, Germany) and maintained at 25 °C. A pre-column with the same packing material was also used. The flow rate was 0.7 mL min⁻¹ and gradient elution was performed with two eluents. Eluent A was 2 mM formic acid and 5 mM ammonium formate in 20% deionized water and 80% acetonitrile; eluent B was 10 mM formic acid and 10 mM ammonium formate in deionized water. The gradient was as follows: column equilibration with 0% eluent B

until 20 min, then linear gradient to 100% B until 35 min, followed by isocratic elution with 100% eluent B until 45 min and finally return to initial 0 % eluent until 46 min. Activity in the *Rhodomonas* bioassay was found in the fraction with retention time of 7 to 9 min. To reduce the sample complexity, the retention time of the collected fraction was narrowed to between 7.5 and 8.5 min. And the dry mass equivalent of the residue was measured.

2.3 Triple quadrupole and Orbitrap mass spectrometry

The LC conditions were the same as for the C8 HPLC or HILIC separation procedures. Triple quadrupole experiments were performed on an API 4000 QTrap instrument (Applied Biosystems, Darmstadt, Germany) equipped with a Turbo ion-spray source. The instrument was operated in the full scan mode in the mass range of m/z 200-2800. Data were acquired with Analyst software 1.4 (Applied Biosystems).

High resolution full scan mass spectra were acquired on Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), coupled to an Ultimate 3000 series RSLC system (Dionex, Idstein, Germany). Fractions were introduced into the mass spectrometer by C8 HPLC using an increasing acetonitrile or methanol gradient at a flow rate of 0.2 mL min^{-1} . Eluent A comprised deionized water containing 0.1% formic acid (FA) for acquisition in positive ESI-mode or ammonium hydroxide ($10 \mu\text{L L}^{-1}$, pH 8) for acquisition in negative ESI-mode, while and Eluent B was acetonitrile and methanol, respectively, containing 0.1% FA/ ammonium hydroxide ($10 \mu\text{L L}^{-1}$, pH 8). The eluents were linearly mixed in a gradient from 5% to 100% B in 15 min, holding 100% B for 12 min and decreasing to 5% B in 1 min of the run. The analytical column was immediately re-equilibrated for 10 min. ESI-MS analysis was performed in positive and negative mode. Full scan mass spectra were generated using 30000 resolving power (FWHM) in the mass range from 200 to 2000 m/z . All peaks in the spectra with $S/N > 3$ were compared between Alex2 and Alex5 fractions using 1 min spectral averaging windows.

2.4 Matrix-assisted laser desorption/ionization Time-of-flight (MALDI-TOF) mass spectrometry

The three different separated and fractionated samples measured by MALDI-TOF were as follows: (1) SPE 80% methanol fractions from 200 mL *A. tamarensis* supernatant; (2) 10 runs pooled HPLC C8 fractions collected from 18 to 19 min; (3) one run of HILIC fractions from 7.5 to 8.5 min. For all the three fraction types, corresponding Alex5 fractions served as a negative control.

A MALDI Micro MX mass spectrometer (Waters, Milford, MA, USA) was used for measurements of compounds in the lower and high mass range between 500-3000 and 2000-40000 m/z in reflectron and linear setup in positive and negative ion modes, respectively. The lyophilized fraction was reconstituted in 100 μL aqueous 0.1% TFA. 1 μL of sample was mixed with 1 μL aliquot of sinapic or dihydrobenzoic acid matrixes (10 mg mL^{-1} in deionized water/acetonitrile 6:4 v/v), and 1 μL of the solution was spotted on a metal 96-spot MALDI target plate. A nitrogen laser (337 nm) was used for ionization, and the extraction of ions was delayed by 500 ns. In positive ion mode, the instrument was operated with 3.5 kV set on the sample plate, -12 kV on the extraction grid. For positive mode reflectron measurements, a reflectron voltage of 5.2 kV was used, the pulse voltage was set to -1.9 kV and the detector was at 2.35 kV with laser energy at 90 $\mu\text{J pulse}^{-1}$. For linear positive ion measurements, the pulse voltage was 800 V, laser intensity was 124 $\mu\text{J pulse}^{-1}$, and the detector voltage was set to 2.15 kV. In negative ion mode measurement, the plate was set to -3.5 kV, the extraction was at 1.2 kV; in reflectron negative measurements, the pulse was 1.95 kV, the detector was set to 2.25 kV with a negative ion acceleration anode set to 4 kV and laser energy was 135 $\mu\text{J pulse}^{-1}$. In linear negative ion measurements, a pulse voltage of 0.75 kV was used and the laser energy was 135 $\mu\text{J pulse}^{-1}$. MassLynx v4.0 software served for data acquisition (Waters). Each spectrum was combined from 15 laser pulses. Insulin, myoglobin and trypsinogen (Sigma) at 10

pmol on target were used to calibrate the mass spectrometer in linear positive mode; a BSA digest at 1 pmol was used to calibrate the instrument in reflectron positive mode.

2.5 Chemical characterization of the lytic compounds

2.5.1 Total sugar quantification

Total sugar content in both Alex2 and Alex5 HILIC fraction (10 runs pooled, dried and re-suspended in 1.6 mL deionized water) were quantified via a modified phenol-sulfuric acid assay (McKelvey and Lee, 1969). A 0.5 mL sample was mixed well with 0.3 mL 6% phenol, and then 1.8 mL concentrated sulfuric acid was added. The mixture was vortexed for 15 s, and then left to cool to room temperature, and the absorption at 480 nm was measured. The sample was calibrated against a D-glucose (Merck, Darmstadt, Germany) standard curve generated with concentrations of 0, 2, 5, 10, 20, and 50 $\mu\text{g mL}^{-1}$. The sugar content in both Alex2 and Alex5 fractions was calibrated against the standard curve.

2.5.2 Trypsin digestion assay and size-exclusion chromatography (SEC)

The lytic HILIC fraction was re-suspended in 1 mL of the trypsin stock solution (total 10 mL), including 2 mg mL^{-1} trypsin (from porcine pancreas, 40U mg^{-1} , Merck, Darmstadt, Germany) in 100 mM NH_4HCO_3 , pH 8.0. As a negative control, an aliquot of 5 mL trypsin from the stock solution was deactivated at 100 °C for 30 min. Before application to the lytic compounds, the trypsin activity was measured with a chromogenic substrate BAPNA (N-benzoyl-DL-arginine-4-nitroanilide hydrochloride, Applichem, Darmstadt, Germany) according to Erlanger et al. (1961). Briefly, ca. 2 mg BAPNA was dissolved in approximately 500 μL dimethylsulfoxide (DMSO) as substrate stock solution. For each test, 960 μL universal buffer (pH 8.0, Stauffer, 1989) with 20 μL enzyme solution was mixed in a cuvette, and read at 405 nm for 5 min at room temperature, and then 20 μL BAPNA substrate was added, mixed well, and then read at 405 nm for 5 more minutes. The enzyme activity was expressed as DA405 nm min^{-1} (change of absorbance at 405 nm per minute). The re-suspended HILIC

fractions in both intact and deactivated trypsin digestion solution were incubated at 25 °C for 18 h. For HILIC fractions in normal enzyme solution, 20 µL sub-samples were taken for an enzyme activity check as described above. All the residues were dried under N₂ and re-suspended in either 1.0 or 0.98 mL K-medium, and stored for two days. A four-point (0.5, 0.25, 0.1, and 0.05 mL) *R. salina* bioassay in 1 mL was performed to quantify the lytic activity in each treatment.

SEC was performed on an Agilent 1100 series (Agilent Technologies, Waldbronn, Germany) system. The LC-system consisted of a G1379A degasser, a G1311A quaternary pump, a G1229A autosampler, a G1330B autosampler thermostat, a G1316A column thermostat and a G1315B diode-array detector (DAD). Chromatographic conditions were as follows: mobile Phase A: 0.1 M Na₂SO₄ and 0.05% NaN₃ in 0.1 M sodium phosphate buffer with pH 6.7. The flow rate was 0.35 mL min⁻¹ until 18 min (= total run time). The autosampler temperature was set to 25 °C and the injection volume was 50 µL. The separation of analytes was performed on a 30 cm × 4.6 mm i.d., TSK-GEL[®] SUPER SW 2000 column (Tosoh Bioscience, Stuttgart, Germany). Chromatograms were recorded at the absorbance wavelength of 280 nm. A spin-filtered lytic SPE fraction was injected into the SEC system. One-minute fractions were collected and checked for lytic activity against *R. salina*. Based on preliminary results, the lytic activity eluted between 10 and 12 min. 200 µL of the spin-filtered SPE fraction were dried under N₂, and digested with 1 mL trypsin solution (0.5 mg mL⁻¹ in 100 mM NH₄HCO₃) or only 100 mM NH₄HCO₃ buffer for 18 h at 25 °C. After 18 h digestion, all the samples were dried under N₂, and re-suspended in 100 µL H₂O. Five one-minute fractions from 9.5 to 14.5 min were collected and dried under N₂, and re-dissolved in 1 mL seawater. Lytic activity was checked in the *R. salina* bioassay. 0.2 µg bovine serum albumin residue (from 100 µL of 2 g L⁻¹ albumin standard stock solution, BCA Protein assay kit (Pierce, Thermo scientific, Rockford, USA)) was treated with 1 mL either trypsin solution or NH₄HCO₃ to examine the activity of trypsin.

3. Results and discussion

3.1 Candidate masses for lytic compounds

The stability of toxins and other allelochemicals, or more precisely the stability of the biological effect quantified by the bioassay, is an important prerequisite for the treatment of the compounds during further analysis and purification. Some compounds degrade rapidly with time and under exposure to light, e.g. toxic compounds of the haptophyte *Prymnesium parvum* (Parnas et al., 1962; Shilo, 1981). Putative toxins produced by the dinoflagellate *Pfiesteria piscicida* are also highly labile, which makes the identification of the compounds difficult (Moeller et al., 2007). In other cases, activity may simply be lost due to surface binding properties during extraction and purification. For example, in preliminary studies of lytic compounds produced by *A. tamarensis*, the activity was often lost or disappeared after the initial steps for isolation and purification by classical bioassay-driven fractionation. (Ma et al, 2009). Further experiments to test the stability of the lytic compounds, however, exhibited high stability of extracellular lytic compounds from *A. tamarensis* under moderate conditions (e.g., normal room temperature and ambient light). In some cases, the recovery of activity after shaking in aqueous solvents indicated that the reduction of activity was not due to degradation of the compounds, but more likely an indication of a very high binding capacity to surfaces. Application of lytic compounds in sufficiently high amounts to compensate for these losses allowed for chromatographic separation on reversed phase and HILIC phases and detection of lytic activity in the corresponding fractions.

3.1.1 Unique masses search with triple quadrupole and orbitrap full scan mass spectrometry

We did not find any unique peaks within the retention time window of Alex2 or Alex5 fractions by either triple quadrupole or orbitrap mass spectrometry. For triple quadrupole mass spectrometry, the fact that no unique masses were found may be due to the low sensitivity under full scan mode. However, in the course of the purification

of lytic fraction on the C8 phase, components of the lytic SPE fractions did not elute well, but rather were retained on the stationary phase and slowly migrated through the column during consequent runs. This resulted in high background noise of the mass spectra and thus hampered the identification of specific masses. When such a fraction was analyzed by orbitrap mass spectrometry, and further chromatographed with the very same C8 column, the high background signal might have interfered with determination of unique masses from the lytic strains. The introduction of an alternative mass spectrometry method, MALDI-TOF, involved no further liquid chromatography.

3.1.2 Unique masses found in SPE fractions

In the beginning of search for lytic compounds excreted by *A. tamarense*, our attention was drawn to two small molecules detected by MALDI-TOF reflectron mode, operating with a resolution of >10000 FWHM (full width at half maximum), analysis of the lytic 80% methanol SPE fraction of Alex2. This highly potent *Alexandrium* strain yielded unique masses at 1291.6 Da and 1061.6 Da, which were absent in the non-lytic strain Alex5 (data not shown).

Such a mass range for the unknown allelochemicals is generally consistent with that of the known phycotoxins, secondary metabolites produced by marine phytoplankton, typically with masses <3000 Da. The only exception is maitotoxin with a molecular mass of 3422 g mol⁻¹, the largest non-biopolymer natural product known (Murata et al., 1994). Karlotoxins from the dinoflagellate *Karlodinium veneficum* (Bachvaroff et al., 2008; Van Wagoner et al., 2008) as well as amphidinols from *Amphidium klebsii* (Houdai et al., 2004; Houdai et al., 2005) have a similar mass range of about 1300 Da and membrane disruptive properties. These compounds form hairpin structures suggested to be important for the interaction with lipid bilayers of biomembranes. The masses of 1291.6 and 1061.6 from the lytic *A. tamarense* strain fall within this mass range. Moreover these compounds, like karlotoxins, are eluted with 80% methanol from C18 SPE cartridges (Bachvaroff et al., 2008). As a working

hypothesis it was reasonable to consider that these two masses may be related to lytic activity and structurally similar to karlotoxins. Nonetheless this hypothesis had to be tested in further experiments.

3.1.3 C8 chromatography

Subsequently, we further purified the lytic SPE 80% MeOH fraction by reversed phase HPLC on a C8 column. Under these chromatographic conditions activity eluted from 18 to 19 min. However, the ion traces of m/z 1061.6 and 1291.6 in a LC-MS experiment showed peaks at retention times of 16 and 15 min, respectively (Fig. 1). Thus those two masses were not related to lytic activity, and thus were excluded as lytic compounds.

Since small molecules could not be correlated to lytic activity, as no other small unique masses were found by LC-MS spectrometry (either triple quadrupole or hybrid linear trap-orbitrap mass spectrometry), we examined a larger mass range, accessible by matrix assisted laser desorption ionization-time of flight (MALDI-TOF/MS) mass spectrometry in the linear mode, by acquiring masses in the range 2000-40000 Da. Thus we prepared lytic C8 HPLC fractions and performed MALDI-TOF analysis in the positive linear mode. The result showed several unique large masses over 7000 Da in the lytic fraction of the Alex2 strain, which were absent in the corresponding fraction of the non-lytic Alex5 strain (Figs. 2A and B). However, the negative mode mass spectra of both Alex2 and Alex5 were almost identical, including an abundant compound with a mass of about 9400 Da (Fig. 2C and D). As mentioned before the lytic C8 fractions were not pure. Reversed phase chromatography on C8 or C18 phases does not seem to be a suitable system for separation and purification of *Alexandrium* lytic compounds because of the non-specific retention of other sample components. Our selection of hydrophilic interaction liquid chromatography (HILIC) as a modified normal phase chromatographic system was assumed not to retain the interfering components that cause problems on reversed phase systems and additionally is compatible with mass spectrometry.

3.1.4 Mass analysis of HILIC fractions

In contrast to reversed phase the lytic compounds separated well on the HILIC system and had a retention time from 7 to 9 min. We also searched for the two former candidate m/z 1062.6 and 1292.6 Da. As expected these compounds eluted at a different retention time (between 6 and 7 min) (Fig. 3) and like on C8 did not co-elute with lytic activity. This independently confirmed our previous finding that the m/z of 1062.6 and 1292.6 Da are not correlated with lytic activity. Even though we narrowed the collection time of the lytic fraction for MALDI-TOF analysis to 7.5 to 8.5 min to minimize the amount of co-eluting contamination, visible precipitation was observed in the dried fractions of the negative control, the corresponding Alex5 fractions. This indicates that even after two clean-up steps with complementary chromatographic systems, the lytic fraction was still not pure, or that Alex5 also produces co-eluting compounds that lack lytic activity due to minor differences in molecular structure. MALDI-TOF analysis in the reflectron mode did not show any masses in the small mass range less than m/z 2000, which were exclusively present in the lytic Alex2 strain, but absent in the non-lytic strain Alex5. For this reason we extended the mass range by using MALDI-TOF in the linear mode with mass coverage from m/z 2,000 to 50,000. In the positive mode, a series of large molecular clusters with masses around 7843, 9746, 11553, 12600, 15020 Da were found in the Alex2 fraction, while no corresponding peaks were found in the Alex5 fraction (Fig. 4A and B). In the negative mode, no characteristic masses could be detected (Fig. 4C and D).

3.2 Chemical nature of the lytic compounds

Thus far the compounds in the mass range between 7 and 15 kDa were the only ones that correlated with lytic activity and were also absent in the non-lytic strain Alex5. This is in accordance with a hemolytic exotoxin over 10 kDa, which was found in *A. taylori* (Emura et al., 2004). Since the lytic compounds are large molecules, at least in relation to the major groups of known phycotoxins, we suspected that they may comprise macromolecular complexes including elements of

the following groups of natural compounds: proteins, polysaccharides, or glycoproteins. Accordingly we set up some assays to test whether or not these elements were present as major constituents of the lytic compounds.

3.2.1 Total sugar content

Total sugar content of corresponding fractions of both, Alex2 and Alex5, was measured and compared. The dry mass equivalent of HILIC Alex2 and Alex5 fractions were 735 ± 227 and 763 ± 110 μg , respectively, and total sugar in each fraction was 0.18 ± 0.01 % and 0.06 ± 0.02 %. The Alex2 HILIC fractions contained about 2-fold more sugar than Alex5 fractions. From the sugar content alone, we cannot exclude or verify that the lytic compounds contain sugar residues. Nevertheless, sugar residues even if present in the structure of the lytic compounds, could only comprise a very small portion of the structure.

3.2.2 Trypsin digestion and SEC

Since many large biomolecules and also the hemolytic compound from *A. taylori* are proteinaceous (Emura et al., 2004), we tested if *A. tamarensis* lytic compounds belong to this chemical class by tryptic digestion. Trypsin cleaves proteins at the carboxyl side of arginine or lysine. Thus compounds with peptides containing these two amino acids can be digested. If the lytic compounds are proteinaceous, they would be digested into smaller peptides, either resulting in smaller size fragments and/or most likely in activity loss. However, the activity of the lytic compounds was not reduced by incubation with trypsin compared to the positive control (incubation of the lytic fraction with deactivated trypsin) (Fig. 5). The EC_{50} in the *Rhodomonas* bioassay of the HILIC fractions treated with at 100 °C deactivated trypsin was 30% (21 - 43%), while no significant differential decrease was detected in the trypsin digested group with EC_{50} of 36% (26 - 53%). For the normal trypsin group, the activity of trypsin against substrate BAPNA was 0.3569 and 0.2449 dA405nm min^{-1} before and after lytic fraction digest assay, respectively, indicating that the enzyme trypsin was active during the digestion procedure.

Although trypsin cannot remove the lytic activity, there still exists the possibility that lytic compounds contain proteinaceous structures, which are not essential for an “active domain” of the activity. To test this hypothesis we used size-exclusion chromatography (SEC), which separates compounds according to their molecular size. If our hypothesis that lytic compounds contain proteinaceous components not essential for lytic activity was correct, the retention time would be increased after tryptic digest. But the first step was to make sure that the lytic compounds could be eluted from the SEC column. According to former experience that the lytic compounds are easily adsorbed to many kinds of materials (Ma et al, 2009), SPE fraction with relatively high activity was applied to the column. Lytic activity eluted from 10 to 12 min (data not shown).

Based on this, the retention time and activity of the SPE fraction after trypsin treatment was compared to the untreated sample. Albumin served as a positive control of trypsin activity and the capacity of the SEC column. The retention time of albumin (ca. 66 kDa) was 8 min (Fig. 6A), and after the tryptic digest the albumin peak disappeared and several small peptide fractions with longer retention time appeared instead (Fig. 6C), indicating that the trypsin digestion worked during incubation. However, in the retention time of lytic activity of SPE fraction, no absorption peak was observed (Fig. 7B), which showed a chromatogram identical to the blank (Fig. 7A). No small peptides peaks appeared after treatment with the trypsin from the same stock applied to albumin (Fig. 7D). The lytic activity in the fractions treated with retention time from 9.5 to 14.5 min were checked, and the result showed neither a retention time shift of lytic activity nor a reduction of lytic activity (Fig. 8). From these results we concluded that although the lytic compounds are large molecules, there is no proteinaceous structure involved.

The SEC experiments additionally confirmed that the lytic compounds are large molecules. The retention time of albumin (66.7 kDa) and trypsin (23.3 kDa) were at 7.8 min and 9.7 min, respectively. The molecular weight of the lytic compounds with

retention time from 10 to 12 min is estimated to be < 16.5 kDa, because on the SEC column the relationship between retention time and molecular weight falls within the linear range for masses between 10^5 Da and 10^4 Da. Such mass range is consistent with the large candidate masses found in Alex2 through MALDI-TOF positive linear mode. Interestingly, the lytic activity was even significantly higher (first lytic fraction with retention time of 9.5 to 10.5 min) in the trypsin digested sample (Fig. 8). Probably, the trypsin digested some proteinaceous impurities which was co-eluted and combined to lytic compounds. After trypsin was applied, the proteinaceous impurities were digested, and no longer bound to lytic compounds. More free lytic compounds therefore killed more *R. salina* cells compared to the sample without trypsin treatment.

4. Conclusion

We partially isolated a suite of large compounds in the range from 7 kDa to 15 kDa in lytic HILIC fraction, the most purified fractions available until now, and subjected the fractions to MALDI-TOF analysis. The broad spectrum enzyme trypsin cannot digest the lytic activity, suggesting the “active domain” is not protein-related. Additionally, based on the results of tryptic digestion-coupled SEC, we conclude that the lytic compounds are large non-proteinaceous compounds (< 23.3 kDa, trypsin). Total sugar content analysis suggested that the lytic HILIC fraction contained two-fold more sugar than the non-lytic fraction, but the sugar content in the HILIC fraction was low, indicating that the major composition of the lytic compounds not comprised of sugar residues. We concluded that one group of allelochemicals produced by *A. tamarense* are large non-proteinaceous, and probably non-polysaccharide compounds between 7 to 15 kDa. However, unambiguous proof that the lytic compounds correspond to particular high molecular masses is still pending. Future research will be concentrated on further purification of lytic fractions and confirmation of the relationship between the lytic compounds and the large masses found in this study.

References

- Adolf, J.E., Bachvaroff, T.R., Krupatkina, D.N., Nonogaki, H., Brown, P.J.P., Lewitus, A.J., Harvey, H.R., Place, A.R., 2006. Species specificity and potential roles of *Karlodinium micrum* toxin. *Afr. J. Mar. Sci.* 28(2), 415-419.
- Adolf, J.E., Krupatkina, D., Bachvaroff, T., Place, A.R., 2007. Karlotoxin mediates grazing by *Oxyrrhis marina* on strains of *Karlodinium veneficum*. *Harmful Algae* 6(3), 400-412.
- Alpermann, T.J., Beszteri, B., John, U., Tillmann, U., Cembella, A.D., 2009. Implications of life history transitions on the population genetic structure of the toxigenic marine dinoflagellate *Alexandrium tamarense*. *Mol. Ecol.* 18(10), 2122-2133.
- Arzul, G., Seguel, M., Guzman, L., Erard-LeDenn, E., 1999. Comparison of allelopathic properties in three toxic *Alexandrium* species. *J. Exp. Mar. Biol. Ecol.* 232, 285-295.
- Bachvaroff, T.R., Adolf, J.E., Squier, A.H., Harvey, H.R., Place, A.R., 2008. Characterization and quantification of karlotoxins by liquid chromatography-mass spectrometry. *Harmful Algae* 7(4), 473-484.
- Blanco, J., Campos, M.J., 1988. The effect of water conditioned by a PSP-producing dinoflagellate on the growth of four algal species used as food for invertebrates. *Aquaculture* 68, 289-298.
- Cembella, A.D., 2003. Chemical ecology of eukaryotic microalgae in marine ecosystems. *Phycologia* 42(4), 420-447.
- Dahl, E., Lindahl, O., Paasche, E., Throndsen, J., 1989. The *Chrysochromulina polylepis* bloom in Scandinavian waters during spring 1988, in: Cosper, E.M., Bricelj, V.M., Carpenter, E.J. (Eds.), *Novel Phytoplankton Blooms: Causes and impacts of recurrent brown tides and other unusual blooms*. Springer Verlag, Berlin, pp. 383-405.
- Emura, A., Matsuyama, Y., Oda, T., 2004. Evidence for the production of a novel proteinaceous hemolytic exotoxin by dinoflagellate *Alexandrium taylori*. *Harmful Algae* 3, 29-37.
- Erlanger, B., Kokowsky, N., Cohen, W., 1961. The preparation and properties of two new chromogenic substrates of trypsin. *Arch. Biochem. Biophys.* 95, 271-278.
- Fistarol, G.O., Legrand, C., Selander, E., Hummert, C., Stolte, W., Granéli, E., 2004. Allelopathy in *Alexandrium* spp.: Effect on a natural plankton community and on algal monocultures. *Aquat. Microb. Ecol.* 35, 45-56.
- Granéli, E., Hansen, P.J., 2006. Allelopathy in harmful microalgae: A mechanism to compete for resources?, in: Granéli, E., Turner, J.T. (Eds.), *Ecology of harmful algae*. Springer-Verlag, Berlin, Heidelberg, pp. 189-201.
- Granéli, E., Weberg, M., Salomon, P.S., 2008. Harmful algal blooms of allelopathic microalgal species: The role of eutrophication. *Harmful Algae* 8(1), 94-102.

- Hansen, P.J., 1989. The red tide dinoflagellate *Alexandrium tamarense*: Effects on behaviour and growth of a tintinnid ciliate. *Mar. Ecol.-Prog. Ser.* 53, 105-116.
- Hansen, P.J., Cembella, A.D., Moestrup, Ø., 1992. The marine dinoflagellate *Alexandrium ostenfeldii*: Paralytic shellfish toxin concentration, composition, and toxicity to a tintinnid ciliate. *J. Phycol.* 28, 597-603.
- Houdai, T., Matsuoka, S., Matsumori, N., Murata, M., 2004. Membrane-permeabilizing activities of amphidinol 3, polyene-polyhydroxy antifungal from a marine dinoflagellate. *Biochim. Biophys. Acta-Biomembr.* 1667(1), 91-100.
- Houdai, T., Matsuoka, S., Morsy, N., Matsumori, N., Satake, M., Murata, M., 2005. Hairpin conformation of amphidinols possibly accounting for potent membrane permeabilizing activities. *Tetrahedron* 61(11), 2795-2802.
- Igarashi, T., Aritake, S., Yasumoto, T., 1998. Biological activities of Prymnesin-2 isolated from a red tide alga *Prymnesium parvum*. *Nat. Toxins* 6, 35-41.
- Keating, K.I., 1977. Allelopathic influence on blue-green bloom sequence in a eutrophic lake. *Science* 196, 885-887.
- Keller, M.D., Selvin, R.C., Claus, W., Guillard, R.R.L., 1987. Media for the culture of oceanic ultraphytoplankton. *J. Phycol.* 23(4), 633-638.
- Kubaneck, J., Hicks, M.K., Naar, J., Villareal, T.A., 2005. Does the red tide dinoflagellate *Karenia brevis* use allelopathy to outcompete other phytoplankton? *Limnol. Oceanogr.* 50(3), 883-895.
- Legrand, C., Rengefors, K., Fistarol, G.O., Granéli, E., 2003. Allelopathy in phytoplankton – Biochemical, ecological and evolutionary aspects. *Phycologia* 42, 406-419.
- Lewis Jr., W.M., 1986. Evolutionary interpretations of allelochemical interactions in phytoplankton algae. *Am. Nat.* 127(2), 184-194.
- Lilly, E.L., Halanych, K.M., Anderson, D.M., 2007. Species boundaries and global biogeography of the *Alexandrium tamarense* complex (Dinophyceae). *J. Phycol.* 43, 1329-1338.
- Ma, H., Krock, B., Tillmann, U., Cembella, A., 2009. Preliminary characterization of extracellular allelochemicals of the toxic marine dinoflagellate *Alexandrium tamarense* using a *Rhodomonas salina* bioassay. *Mar. Drugs* 7(4), 497-522.
- Matsuoka, K., Cho, H.J., Jacobsen, D.M., 2000. Observation of the feeding behaviour and growth rates of the heterotrophic dinoflagellate *Polykrikos kofoidii* (Polykrikaceae, Dinophyceae). *Phycologia* 39(1), 82-86.
- McKelvey, J.F., Lee, Y.C., 1969. Microheterogeneity of the carbohydrate groups of *Aspergillus oryzae* alpha-amylase. *Arch. Biochem. Biophys.* 132, 99-110.
- Moeller, P.D.R., Beauchesne, K.R., Huncik, K.M., Davis, W.C., Christopher, S.J., Riggs-Gelasco, P., Gelasco, A.K., 2007. Metal complexes and free radical toxins produced by *Pfiesteria piscicida*. *Environ. Sci. Technol.* 41(4), 1166-1172.
- Murata, M., Naoki, H., Matsunaga, S., Satake, M., Yasumoto, T., 1994. Structure and partial stereochemical assignments for maitotoxin, the most toxic and largest natural non-biopolymer. *J. Am. Chem. Soc.* 116, 7098-7107.

- Parnas, I., Reich, K., Bergmann, F., 1962. Photoinactivation of ichthyotoxin from axenic cultures of *Prymnesium parvum*. *Appl. Microbiol.* 10, 237-239.
- Pratt, D.M., 1966. Competition between *Skeletonema costatum* and *Olisthodiscus luteus* in Narragansett Bay and in culture. *Limnol. Oceanogr.* 11, 447–455.
- Prince, E.K., Poulson, K.L., Myers, T.L., Sieg, R.D., Kubanek, J., 2010. Characterization of allelopathic compounds from the red tide dinoflagellate *Karenia brevis*. *Harmful Algae* 10(1), 39-48.
- Rengefors, K., Legrand, C., 2001. Toxicity in *Peridinium aciculiferum* - An adaptive strategy to outcompete other winter phytoplankton? *Limnol. Oceanogr.* 46(8), 1990-1997.
- Rice, E.L., 1984. Allelopathy, 2nd ed. Academic Press, New York.
- Shilo, M., 1981. The toxic principle of *Prymnesium parvum*, In: Carmichael, W.W. (Ed.), *The water environment. Algal toxins and health*. Plenum Press, New York, pp. 33-47.
- Stauffer, C.E., 1989. *Enzyme Assays for Food Scientist*. AVI Press, Van Nostrand-Reinhold, New York.
- Sugg, L.M., VanDolah, F.M., 1999. No evidence for an allelopathic role of okadaic acid among ciguatera-associated dinoflagellates. *J. Phycol.* 35(1), 93-103.
- Suikkanen, S., Fistarol, G.O., Graneli, E., 2005. Effects of cyanobacterial allelochemicals on a natural plankton community. *Mar. Ecol. Prog. Ser.* 287, 1-9.
- Tillmann, U., Alpermann, T., John, U., Cembella, A.D., 2008a. Allelochemical interactions and short-term effects of the dinoflagellate *Alexandrium* on selected photoautotrophic and heterotrophic protists. *Harmful Algae* 7(1), 52-64.
- Tillmann, U., John, U., Krock, B., Cembella, A.D., 2008b. Allelopathic effects of bioactive compounds produced by harmful algae, In: Moestrup, Ø. e.a. (Ed.), *12th International Conference on Harmful Algae*. IOC-UNESCO, Copenhagen, Denmark, pp.12-18.
- Tillmann, U., Hansen, P.J., 2009. Allelopathic effects of *Alexandrium tamarense* on other algae: evidence from mixed growth experiments. *Aquat. Microb. Ecol.* 57, 101-112
- Tillmann, U., Alpermann, T.L., da Purificação, R.C., Krock, B., Cembella, A.D., 2009b. Intra-population clonal variability in allelochemical potency of the toxigenic dinoflagellate *Alexandrium tamarense*. *Harmful Algae* 8(5), 759-769.
- Tillmann, U., John, U., 2002. Toxic effects of *Alexandrium* spp. on heterotrophic dinoflagellates: An allelochemical defence mechanism independent of PSP-toxin content. *Mar. Ecol.-Prog. Ser.* 230, 47-58.
- Tillmann, U., John, U., Cembella, A.D., 2007. On the allelochemical potency of the marine dinoflagellate *Alexandrium ostenfeldii* against heterotrophic and autotrophic protists. *J. Plankton Res.* 29(6), 527-543.
- Van Wagoner, R.M., Deeds, J.R., Satake, M., Ribeiro, A.A., Place, A.R., Wright, J.L.C., 2008. Isolation and characterization of karlotoxin 1, a new amphipathic toxin from *Karlodinium veneficum*. *Tetrahedron Lett.* 49(45), 6457-6461.

- Vardi, A., Schatz, D., Beerli, K., Motro, U., Sukenik, A., Levine, A., Kaplan, A., 2002. Dinoflagellate-cyanobacterium communication may determine the composition of phytoplankton assemblage in a mesotrophic lake. *Curr. Biol.* 12(20), 1767-1772.
- Windust, A.J., Quilliam, M.A., Wright, J.L.C., McLachlan, J.L., 1997. Comparative toxicity of the diarrhetic shellfish poisons, okadaic acid, okadaic acid diol-ester and dinophysistoxin-4, to the diatom *Thalassiosira weissflogii*. *Toxicon* 35(11), 1591-1603.
- Windust, A.J., Wright, J.L.C., McLachlan, J.L., 1996. The effects of the diarrhetic shellfish poisoning toxins, okadaic acid and dinophysistoxin-1, on the growth of microalgae. *Mar. Biol.* 126(1), 19-25.
- Wolfe, G.V., 2000. The chemical defense ecology of marine unicellular plankton: constraints, mechanisms, and impacts. *Biol. Bull.* 198(2), 225-244.
- Yamasaki, Y., Shikata, T., Nukata, A., Ichiki, S., Nagasoe, S., Matsubara, T., Shimasaki, Y., Nakao, M., Yamaguchi, K., Oshima, Y., Oda, T., Ito, M., Jenkinson, I.R., Asakawa, M., Honjo, T., 2009. Extracellular polysaccharide-protein complexes of a harmful alga mediate the allelopathic control it exerts within the phytoplankton community. *ISME Journal* 3(7), 808-817.
- Yasumoto, T., Underahl, B., Aune, T., Hormazabal, V., Skulberg, O.M., Oshima, Y., 1990. Screening for hemolytic activity and ichthyotoxic components of *Chrysochromulina polylepis* and *Gyrodinium aureolum* from Norwegian coastal waters, In: Graneli, E., Sundstrom, B., Edler, L., Anderson, D.M. (Eds.), *Toxic Marine Phytoplankton*. Elsevier, Lund, Sweden, pp. 436-440.

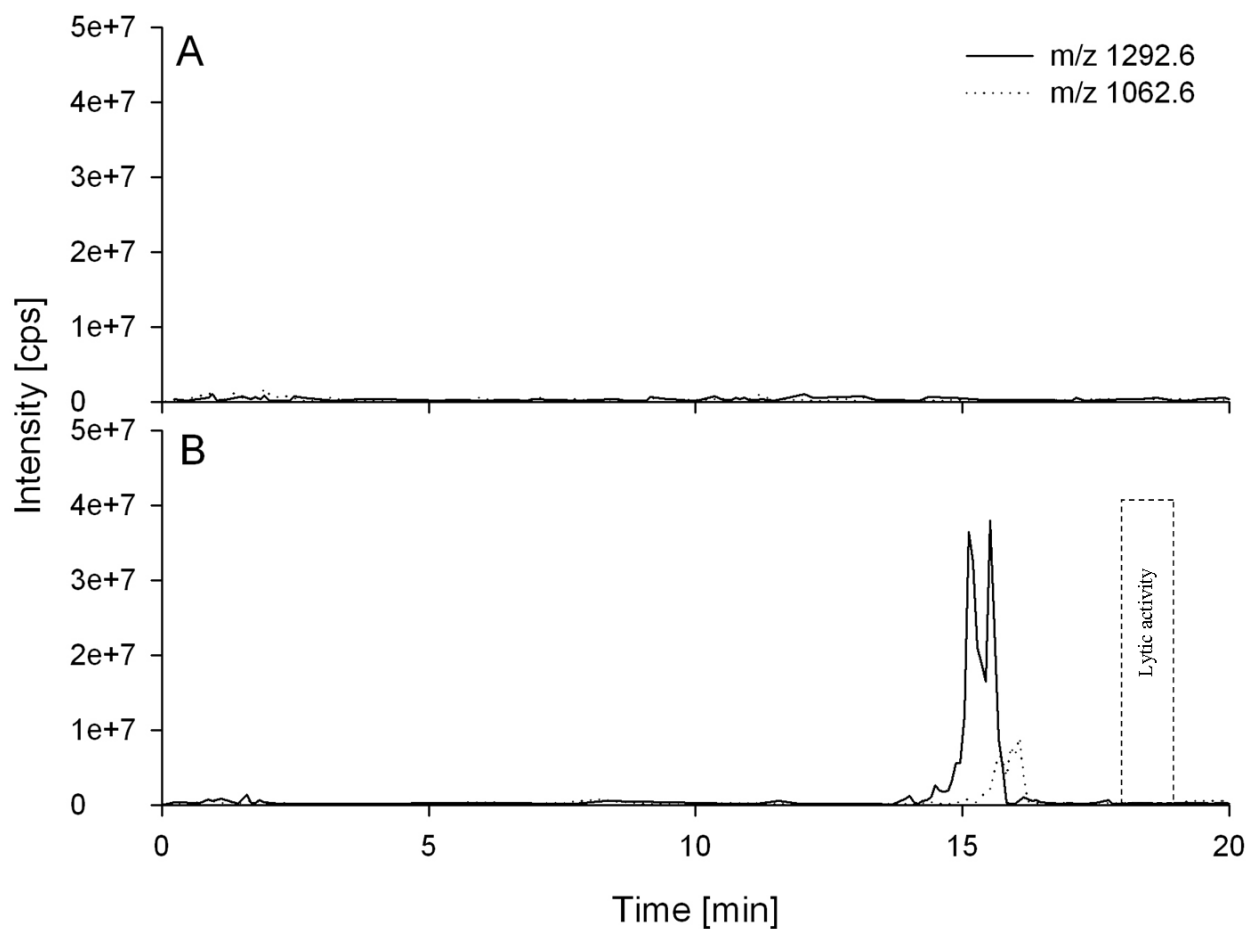


Fig. 1 Retention time of 1291.6 Da and 1062.6 Da peaks in liquid chromatography/triple quadrupole mass spectrum after separation on a C8 column. (A) Alex5; (B) Alex2.

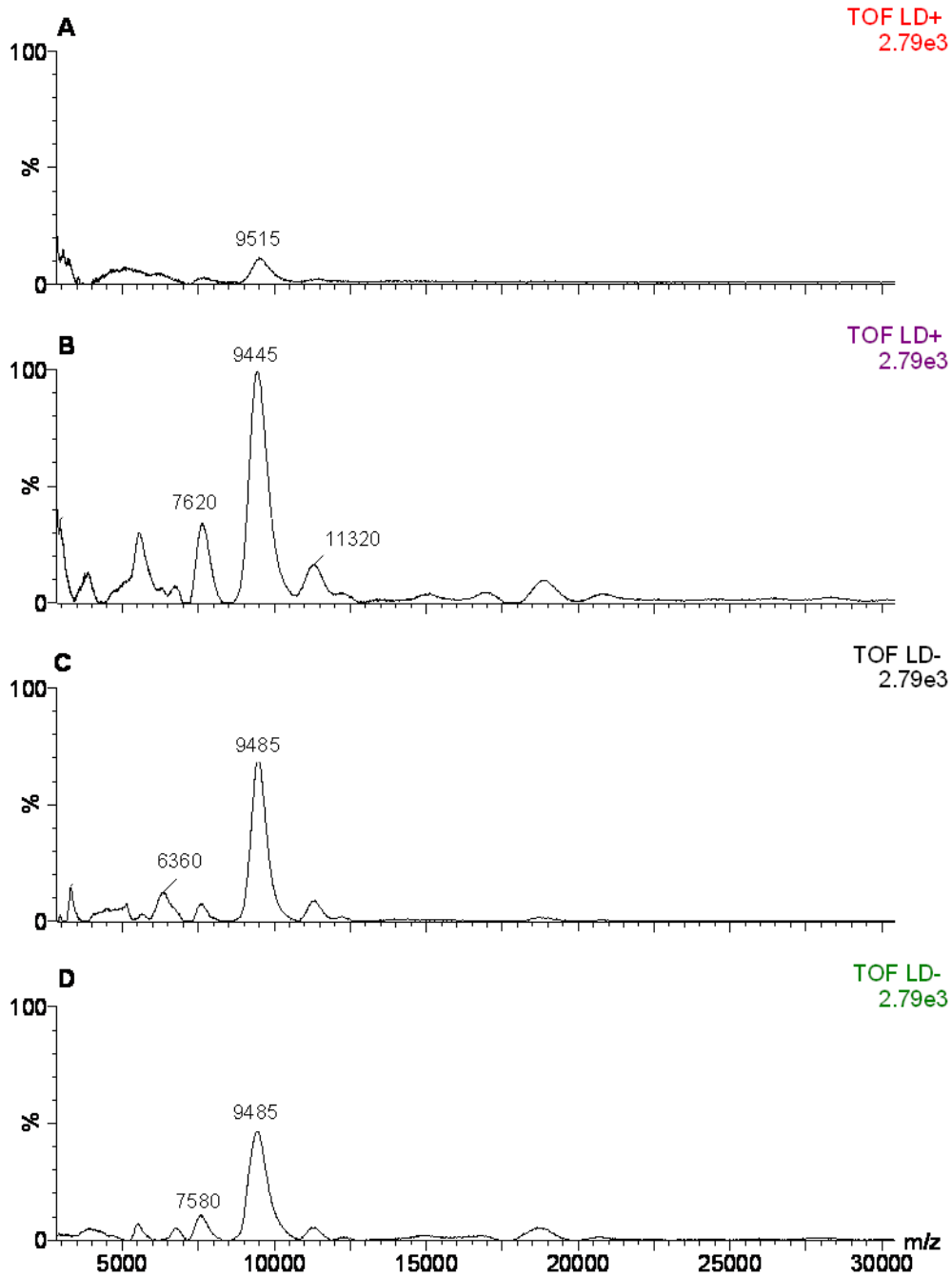


Fig. 2 MALDI-TOF mass spectrum of C8 HPLC fractions. (A) Alex5, linear positive mode; (B) Alex2, linear positive mode; (C) Alex5, linear negative mode; (D) Alex2, linear negative mode.

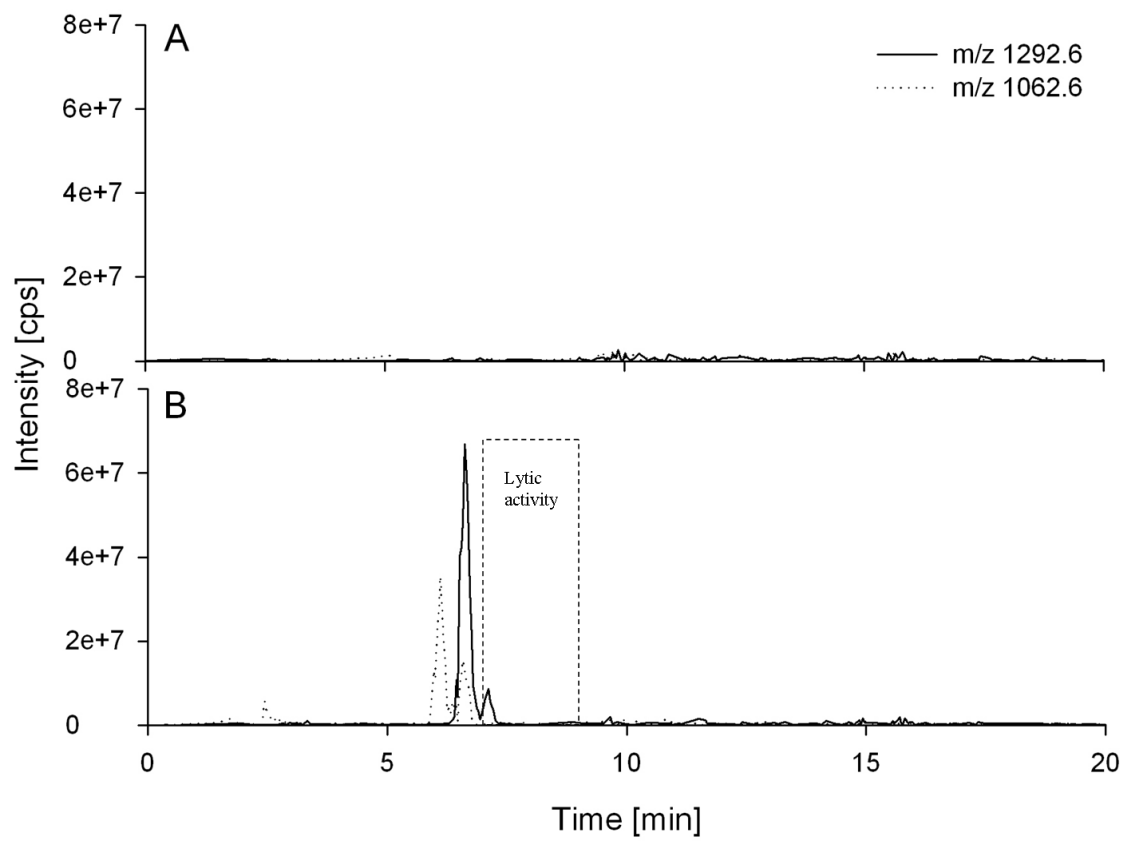


Fig. 3 Retention time of 1291.6 Da and 1062.6 Da in liquid chromatography/ triple quadrupole mass spectrum through ZIC-HILIC column. (A) Alex5; (B) Alex2.

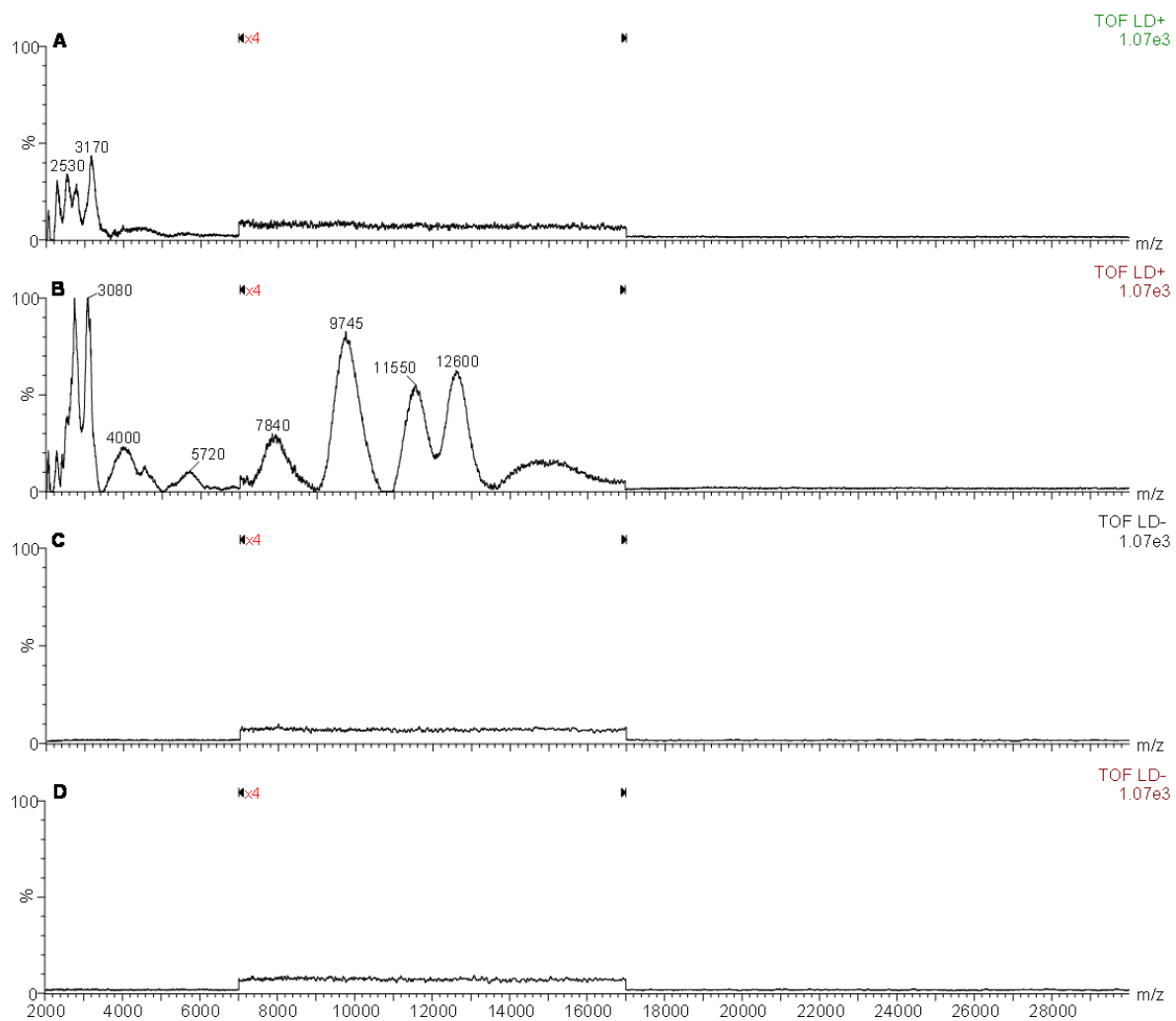


Fig. 4 MALDI-TOF mass spectrum of HILIC fractions. A. Alex5, linear positive mode; B. Alex2, linear positive mode; C. Alex5, linear negative mode; D. Alex2, linear negative mode.

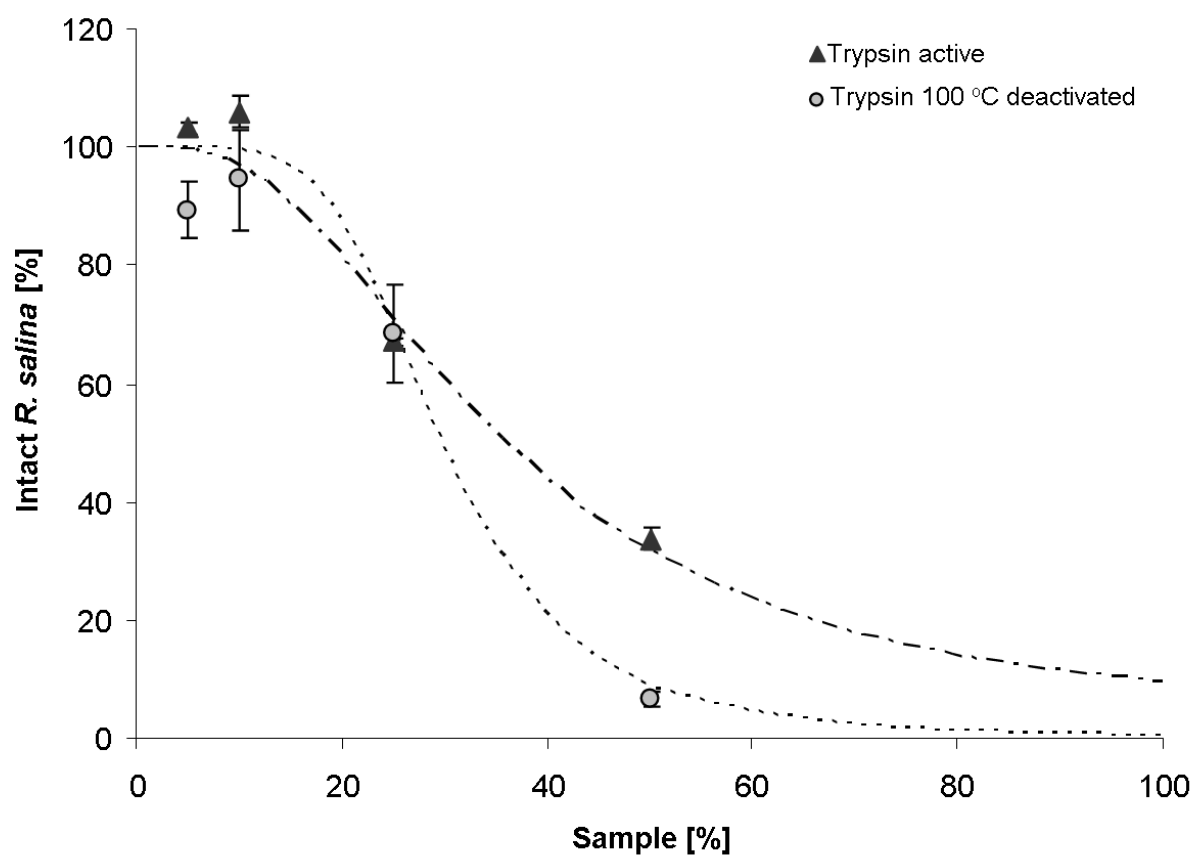


Fig. 5 HILIC active fraction digested with normal or deactivated trypsin. Results expressed as duplicate mean \pm SD.

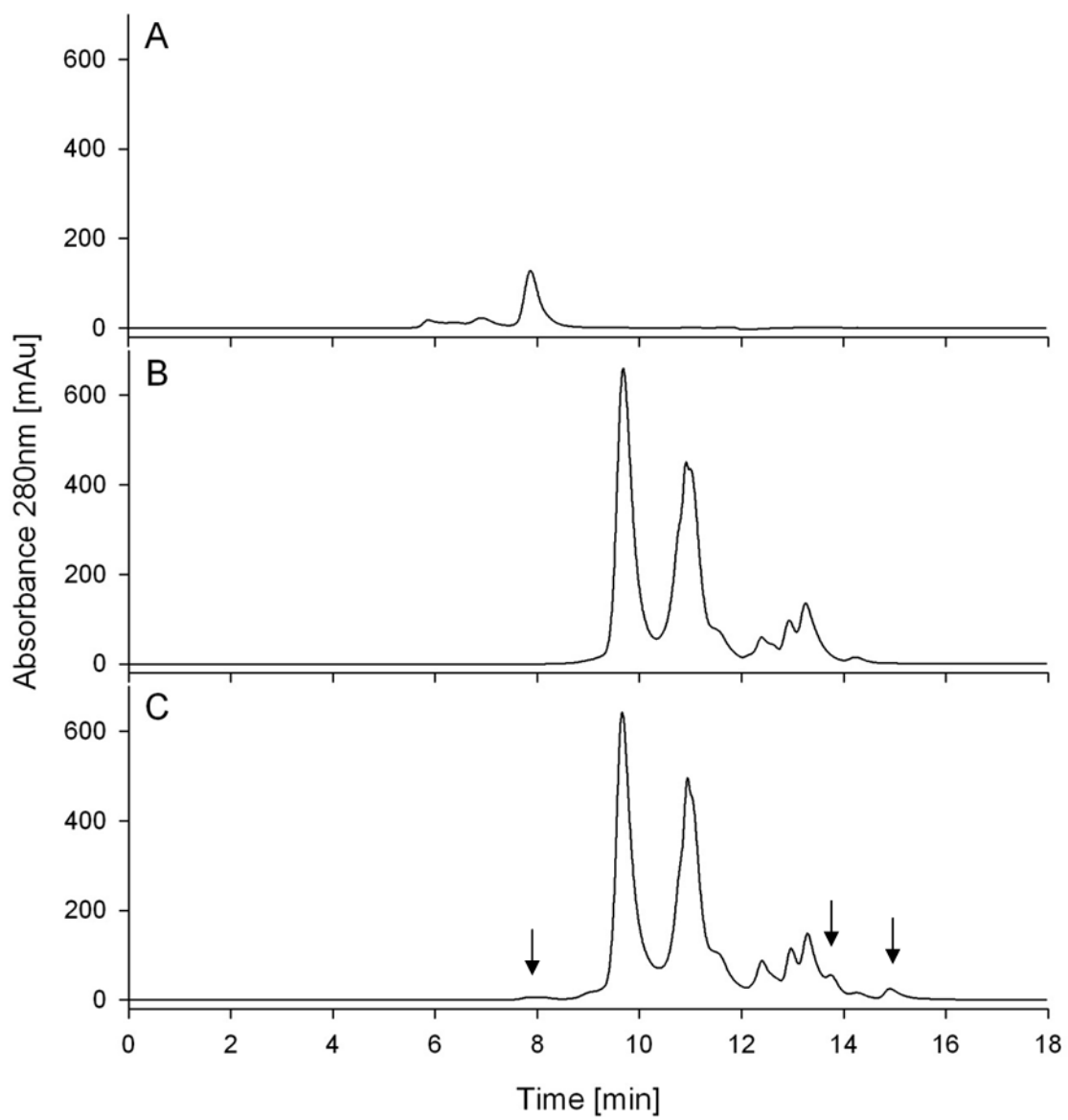


Fig. 6 Size exclusion chromatography of albumin treated with or without trypsin before application to the column. (A) albumin (B) trypsin (C) albumin+ trypsin.

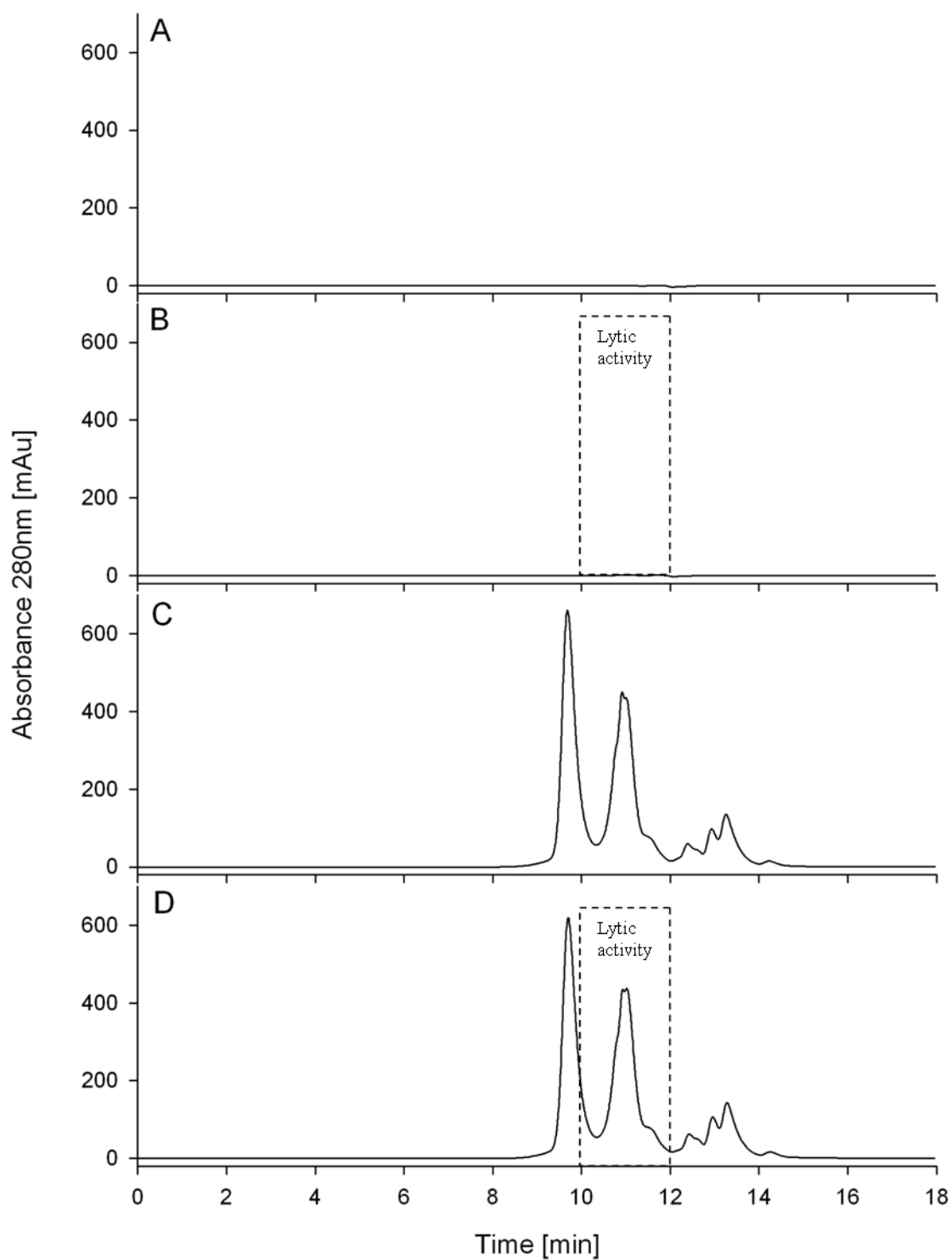


Fig. 7 Size exclusion chromatography of lytic SPE 80% methanol fraction treated with or without trypsin before applied to the column. (A) blank (B) SPE (C) trypsin (D) SPE + trypsin

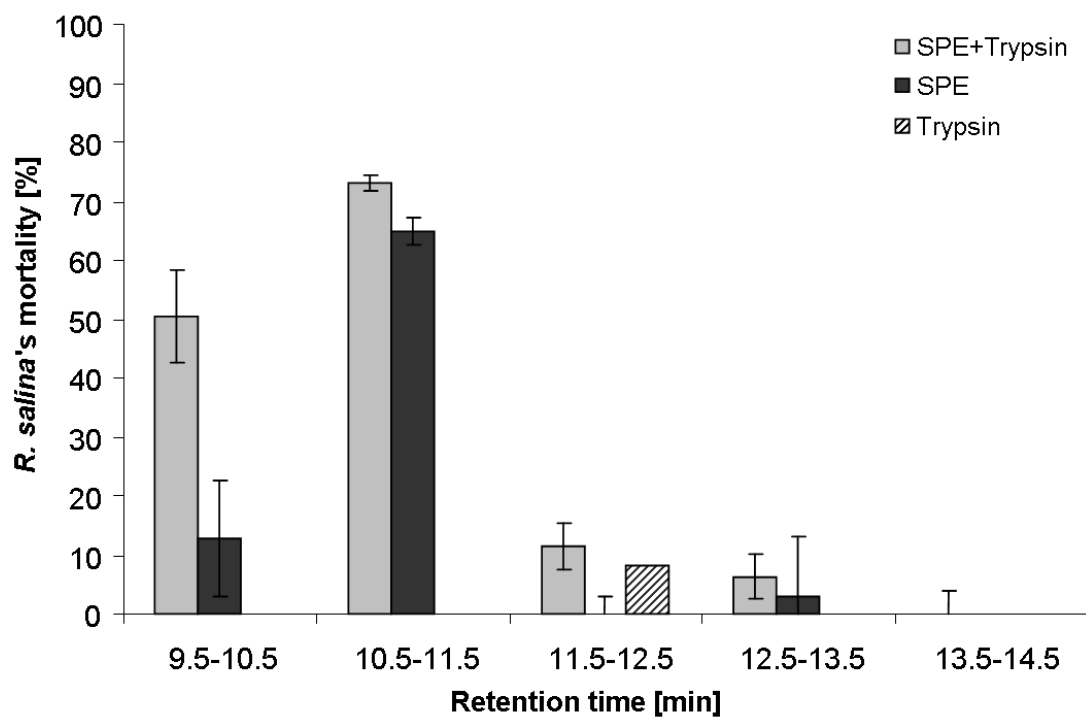


Fig. 8 Retention time and lytic activity of SEC fractions separated from SPE fractions treated with or without trypsin. Results expressed as triplicate mean \pm SD.