The Dehydratase Activity of Lacticin 481 Synthetase is Highly Processive

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Supporting Information

Generation of Enzyme and Substrate

Enzyme and wild-type substrate were prepared as previously described.[1] Isotopically depleted LctA was grown in modified M9 minimal media.[2] BL21(DE3) cells containing the pET15b-LctA plasmid were grown overnight at 37 ºC in LB containing ampicillin (100 μg/mL). After spinning down the cells, the supernatant was discarded and the cells were washed with 5 mL of wash buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 0.5 g NaCl per liter, pH 7.4). Following washing, the cells were resuspended in 2 mL of wash buffer and added to 300 mL of modified M9 minimal media. Modified M9 minimal media contained 30 mL of a 10X stock of minimal media (15 g KH_2PO_4, 30 g Na_2HPO_4, 2.5 g NaCl in 500 mL, pH 7.4), 0.8 mL of 40% $(^{14}NH_4)_2SO_4$ (Sigma-Aldrich), 0.55 g of ^{13}C-depleted glucose (Cambridge Isotope Laboratories), 0.2 mg of $FeSO_4$, 20 μg thiamine, 400 μL of 1 M $MgSO_4$, 20 μL of 1 M $CaCl_2$, 50 μg/mL ampicillin and 150 μL of a trace element solution containing 0.55g $CaCl_2$, 0.17 g $ZnCl_2$, 0.044 g $CuCl_2 \cdot H_2O$, 0.06 g $CoCl_2 \cdot 6H_2O$, 0.06 g $Na_2MoO_4 \cdot 2H_2O$ in 1 L at pH 7.4. The cells were induced at OD_{600} = 0.7 by the addition of IPTG to a final concentration of 0.7 mM and grown for an additional 6 h at 37 ºC before harvesting. The cell pellet (~2 g) was resuspended in 6 mL of start buffer 2 (20 mM Na_2HPO_4, pH 7.5, 500 mM NaCl, 0.5 mM imidazole). The cells were lysed by sonication and then centrifuged. The resulting pellet was resuspended in 6 mL of denaturing buffer (start buffer 2 with 6 M guanidine hydrocholoride) and then sonicated again to redissolve the protein. The remaining insoluble material was pelleted by centrifugation following which the supernatant was mixed with Ni-NTA resin at 4 ºC for 3 h. The resin mixture was loaded onto a column. After allowing the column to drain, the resin was washed with 50 mL of start buffer 2 (with 6 M guanidine hydrochloride) followed by a wash with 50 mL of wash buffer (20 mM Na_2HPO_4, pH 7.5, 300 mM NaCl, 30 mM imidazole, 4 M guanidine hydrochloride). The peptide was eluted four times with 5 mL of elute buffer (20 mM Na_2HPO_4, pH 7.5, 1 M NaCl, 50 mM EDTA). The samples containing isotopically depleted LctA were dried on a speed-vac and then resuspended in H_2O containing 0.1% TFA before being injected onto an analytical C4 column. The peptide eluted at 19.4 min with a gradient of 2-100% B over 40 min (B = CH_3CN/0.1% TFA).

Instrumentation

All data was acquired on a custom-built 8.5 T Quadrupole-FTMS.[3] Samples were introduced via automated nanospray (NanoMate 100, Advion BioSciences) into the instrument. A series of ion optics were used to direct the ions into the trapped ion cell of the Q-FTMS. A quadrupole in front of the cell could be used in mass selection mode to selectively enhance the ions in a window ~50 *m/z* in width. For MS/MS experiments, the ions of interest were isolated in the FTMS cell using a stored waveform inverse Fourier transform (SWIFT; window size ~1 *m/z*), then subsequently irradiated with a 75 W CO_2

laser for 100-200 ms.[4] Data was acquired using a MIDAS data station. The fragment ion prediction program of the ProSight PTM software bundle was used to generate fragment ion masses (https://prosightptm.scs.uiuc.edu).

Time Course of Conversion LctA (natural isotopic abundance) to Product

Assays with 11 µM LctA (natural isotopic abundance), in buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 µM $ZnCl_2$ and 25 µg/mL BSA were incubated at 25 ºC with His_6-LctM at a final concentration of 0.19 µM. The assays were quenched by filtering through a 0.45 µm membrane into 5 µL TFA or 20 mg TCEP after 15 s, 45 s, 75 s and 5 min. The samples were then purified on a C4 analytical HPLC column (the samples with TCEP were incubated for 20 min at 25 ºC prior to HPLC analysis). The fractions were then dried down and resuspended in 30 µL 49% CH_3CN, 49% H_2O and 2% acetic acid for FTMS analysis. The results are shown in Figure S1.

Complete conversion to product is observed after 5 min. Also, evidence for species containing 2-4 dehydrations are observed after 15 and 45 s. The 75 s time point shows species that may be a mixture of 2 and 3 dehydrations along with oxidations of the -4 H_2O species. Using depleted LctA allows for distinct identification of dehydrated species versus species with oxidations (a 2 Da difference). Figure S2 shows the isotopic distributions for LctA with the natural abundance of isotopes and LctA expressed in ^{13}C and ^{15}N depleted media.

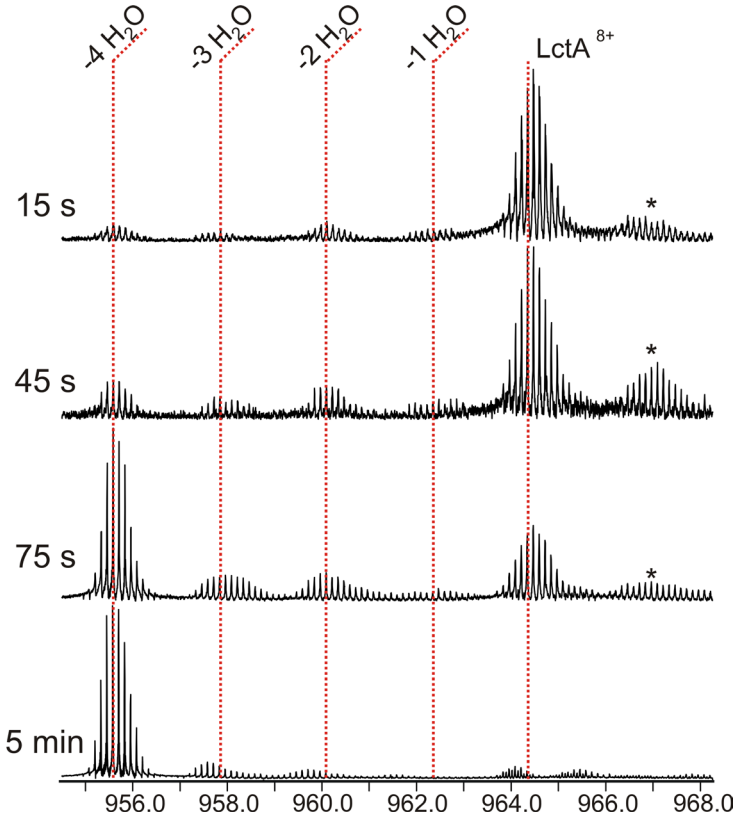

Figure S1. Time course with natural abundance LctA with an enzyme:substrate ratio of 1:60. An * denotes an adduct (oxidation or sodium).

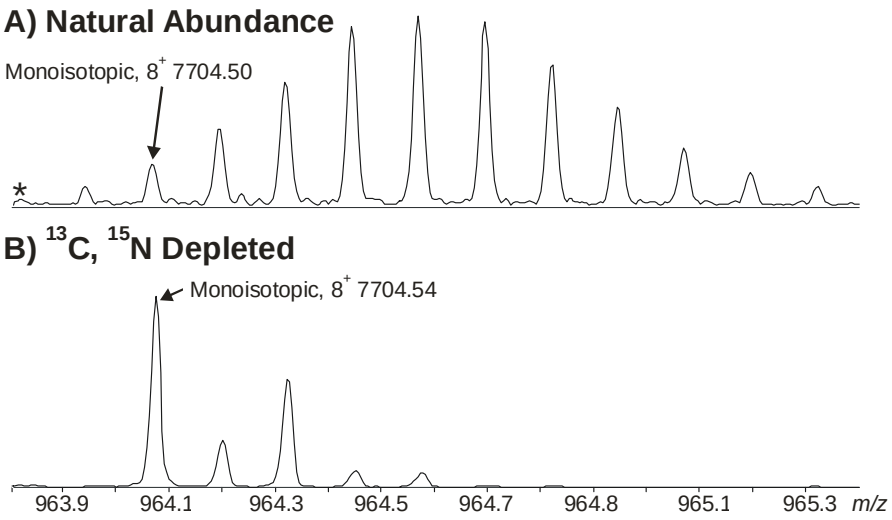

Figure S2. A) The isotopic distribution for natural abundance LctA (8+ charge state). An * indicates the monoisotopic peak for the starting material containing a disulfide, which is 2 Da lower. B) The 8+ charge state for ^{13}C, ^{15}N depleted LctA. In both panels the monoisotopic peak is labeled.

Assays with LctM and ATP

HPLC purified (C4 column) isotope depleted LctA was redissolved in sterile Millipore water to a final concentration of 4 mg/mL (520 μM) as determined by the Bradford assay. The assays with LctM were carried out in a buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 0.5 μM $ZnCl_2$ and 25 μg/mL BSA (Sigma, St. Louis, MO). The substrate, enzyme and ATP concentrations were varied as discussed below. All assays were performed at 25 ºC. After quenching, each reaction mixture was loaded onto a pre-equilibrated C18 ZipTip, washed with H_2O/0.1% TFA, and eluted into 4 μL 78% CH_3CN, 20% H_2O and 2% acetic acid of and then diluted with 30 μL of 49% CH_3CN, 49% H_2O and 2% acetic acid prior to nanospray and FTMS analysis.

Time Course of Conversion of ^{13}C, ^{15}N Depleted LctA to Product

1:1 Enzyme to Substrate

A chemical quench-flow apparatus (Model RQF-3, KinTek Corporation, Austin, TX) was used to obtain the initial time points (50 and 500 ms). An aliquot of 100 μL of a reaction mixture containing 5.2 μM ^{13}C, ^{15}N depleted LctA in the buffer described above with 5 mM ATP (final reaction concentration) was loaded into the first reaction loop and a sample of 100 μL of solution containing 5.2 μM His_6-LctM was loaded into the second. Approximately 100 μL of a 5% TFA solution was used to quench the reactions, giving a final reaction volume of ~300 μL. The final enzyme to substrate ratio for each reaction was 1:1.

For longer time points, His_6-LctM was added to a final concentration of 2.6 μM to a reaction mixture containing 2.6 μM ^{13}C, ^{15}N depleted LctA and 5 mM ATP in the buffer described above. The 200 μL reactions were quenched at the following time points by the addition of 5 μL TFA: 1 s, 3 s, 10 s, 15 s, 25 s, 35 s, 50 s, and 2 min. Each

fraction was run on the 8.5 T Q-FTMS. Figure S3 illustrates the quadrupole enhanced regions of the spectra containing the 7+ charge states. After 50 ms no masses were seen corresponding to any dehydration events. The same was also observed for the 500 ms time point (data not shown). A 3 s time point revealed all five species including those with 1, 2 and 3 dehydrations. The 10 s and 35 s time points show an increasing amount of product (-4 H_2O) and in both cases a species corresponding to 3 dehydrations. After 2 min, no amount of starting material remained and only a species with four dehydrations was observed.

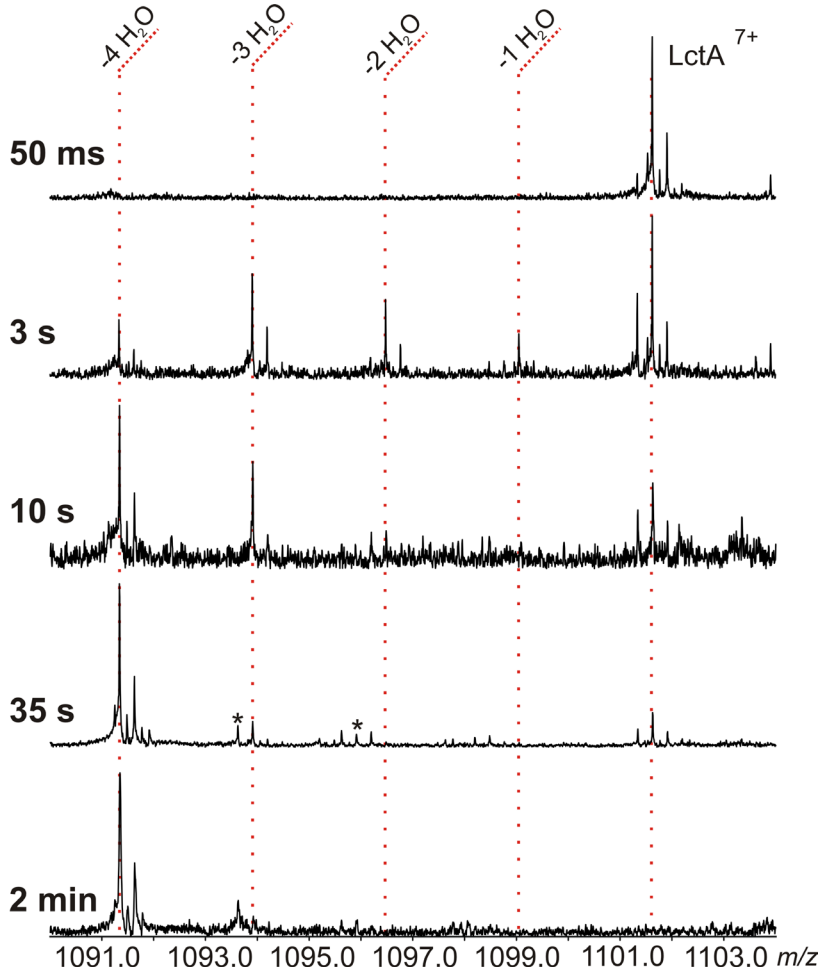

Figure S3. Time course with an enzyme to substrate ratio of 1:1. The region shown contains the 7+ ions for all possible forms of LctA. An * denotes an oxidation product.

1:90 Enzyme to Substrate

A series of time points was performed at an enzyme to substrate ratio of 1:90. His_6-LctM was added to a final concentration of 0.13 µM to a reaction mixture containing 11 µM ^{13}C, ^{15}N depleted LctA and 0.5 mM ATP. The reactions were quenched at the following time points by filtering through a 0.45 µm membrane into 5 µL TFA: 15 s, 45 s, 75 s and 5 min. A control reaction was also done in the same manner as above, except it contained no ATP. Figure S4 shows the quadrupole enhanced regions of the spectra containing the 8+ charge states for all possible forms of LctA for all the time points. After 15 s only a small amount of products appear with three and four

dehydrations. At 45 s more of the material has been converted to a species with a mass consistent with four dehydrations with a small amount of material present with both two and three dehydrations. A similar spectrum is seen after 75 s with a larger amount of material with four dehydrations. After 5 min almost all of the starting material is consumed with the majority of material having a mass consistent with four dehydrations. Small amounts of three and two dehydrations are also seen.

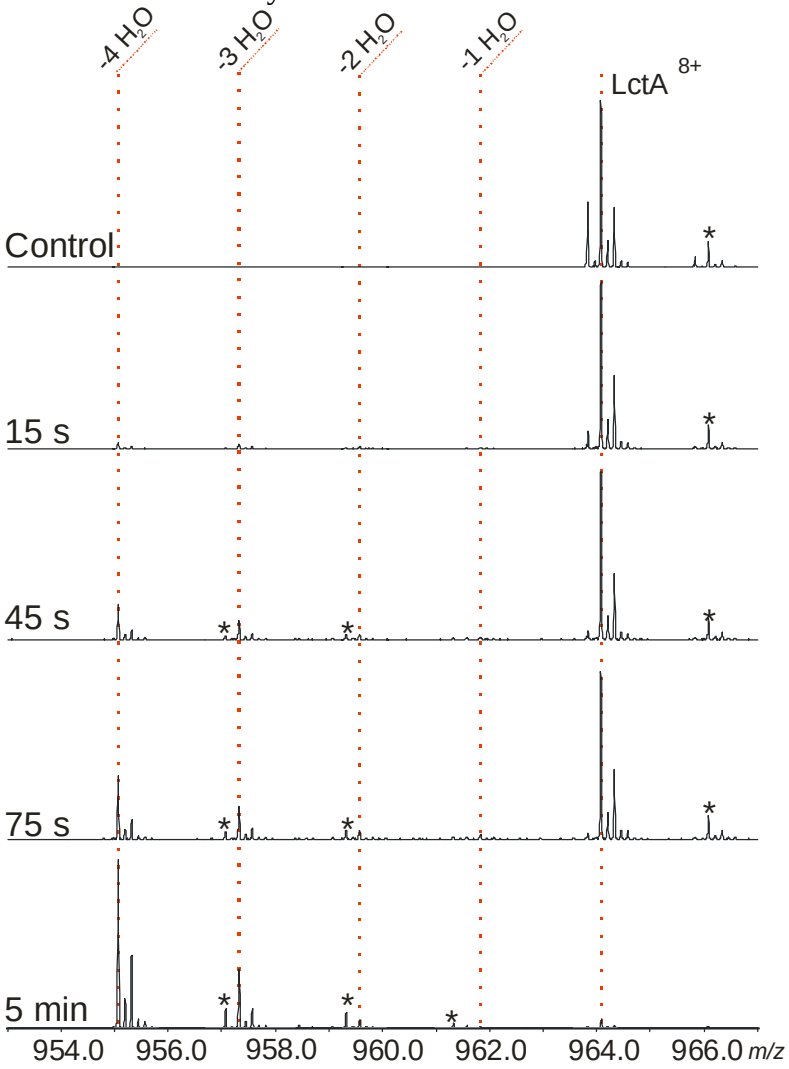

Figure S4. Time course with an enzyme to substrate ratio of 1:90. An * denotes an oxidation.

1:900 Enzyme to Substrate

A series of time points was done at an enzyme to substrate ratio of 1:900. His_6-LctM was added to a final concentration of 0.013 µM to a reaction mixture containing 11 µM ^{13}C, ^{15}N depleted LctA and 0.5 mM ATP. The reactions were quenched at the following time points by filtering through a 0.45 µm membrane into 5 µL TFA: 1 min, 3 min, 5 min, 7.5 min, 10 min, 20 min and 40 min. A control reaction, without ATP, was also done at the same enzyme to substrate ratio. Selected time points are shown in Figure 1 of the main article.

Cyanylation of Time Points

A series of time-limited assays of His_6-LctA with His_6-LctM were undertaken and the assay products purified by C4 analytical HPLC. The purified assay products were resuspended in 20 µL of 2.5 mM TCEP, 4 M Gn-HCl in 100 mM citric acid buffer pH 3.0 by vortexing, followed by incubation at 37 °C for 15 min, to minimize the occurrence of disulfides that would prevent cyanylation of non-cyclized thiols. The reduced assay products were then subjected to cyanylation with 20 µL of 200 mM 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) in 100 mM citric acid, pH 3.0 for 15 min at 37 °C.[5] At the end of incubation the samples were acidified with 3 µL of 5% TFA and purified by C18 ZipTip prior to analysis by ESI-FTMS. As a control, His_6-LctA was subjected to identical conditions and the results of cyanylation are as shown in Figure S5.

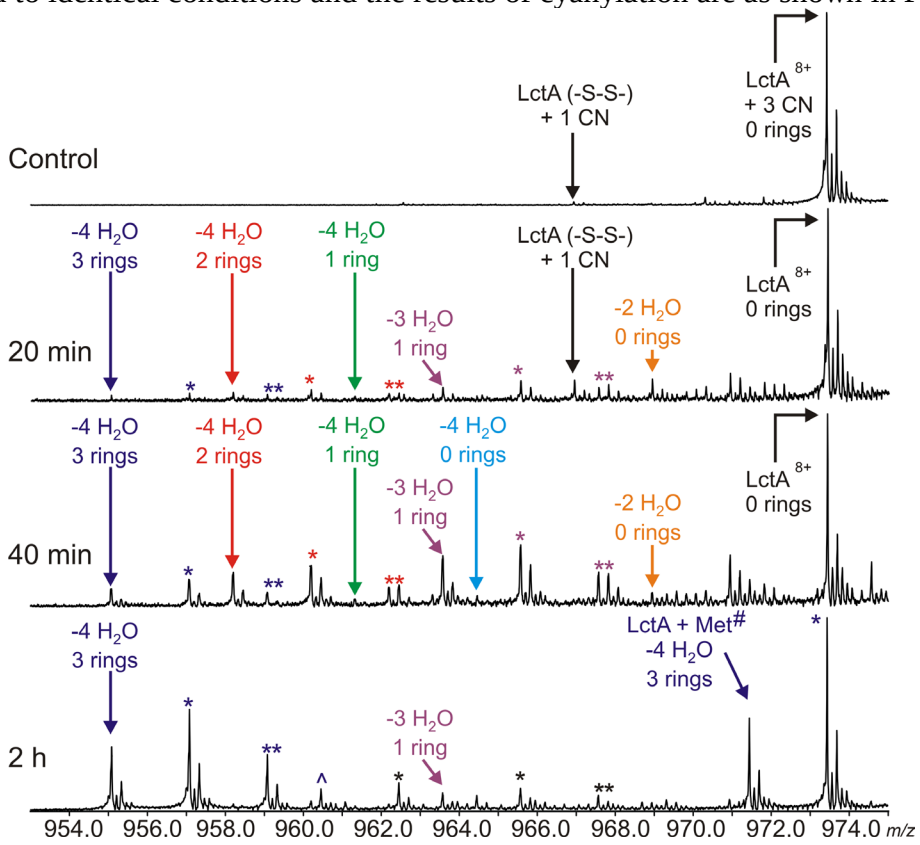

Figure S5. Cyanylation of different time points of 1:900 enzyme to substrate ratio (2 h time point is an enzyme to substrate ratio of 1:90). All species shown are the 8+ ions. An * denotes one oxidation, ** two oxidations and ^ carbamylation. An # denotes a peak arising from incomplete removal of N-terminal Met.

MS/MS of the Starting Material and Product

Both a control experiment and a 2 h time point with an enzyme:substrate ratio of 1:90 were fragmented via IRMPD to determine the presence or absence of the lanthionine rings. It has previously been shown that the regions that are cyclized will not undergo fragmentation.[1] Figure S6A shows the control spectrum, where the starting material was isolated and fragmented. The inset represents the fragment ions generated via IRMPD.

Fourteen b-type and 5 y-type fragment ions were observed, with many of those being in the region which is expected to be cyclized in the presence of LctM. In figure S6B, a spectrum is shown in which the LctA has been converted to product. The inset is the fragment ion map of ions from IRMPD of the species with four dehydrations. Eleven b-type and 19 y-type fragment ions were observed, but none were observed in the region containing the lanthionine rings indicating that LctA had been fully dehydrated and cyclized in 2 h.

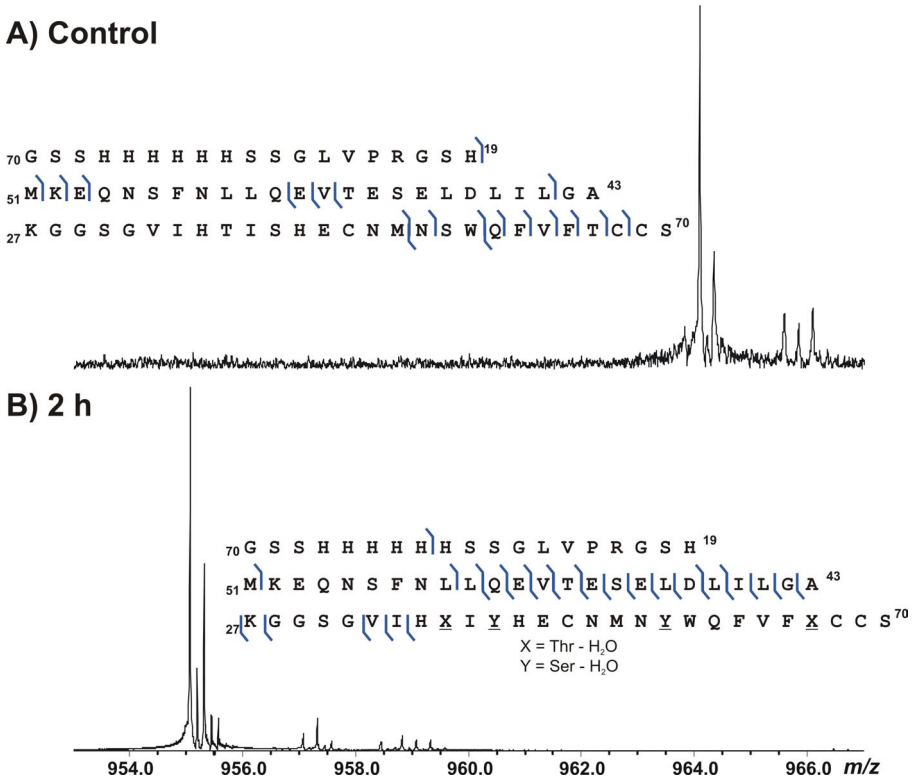

Figure S6. A) A control sample in which the starting material has not undergone any processing. Inset, IRMPD fragment ion map of the starting material. B) Spectrum containing a species with four dehydrations. Inset, fragment ion map of depleted LctA after being treated with LctM (1:90 enzyme to substrate) for 2 h. The data are consistent with formation of all 3 lanthionine rings.

References

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