Accelerated Protein Synthesis *via* One–Pot Ligation–Deselenization Chemistry

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SUMMARY

Peptide ligation chemistry has revolutionized protein science by facilitating access to synthetic proteins. Herein, we describe the development of additive–free ligation–deselenization chemistry at β-selenoaspartate and γ-selenoglutamate that enables the generation of native polypeptide products on unprecedented time scales. The deselenization step is chemoselective in the presence of unprotected selenocysteine, which is highlighted in the synthesis of selenoprotein K. The power of the methodology is also showcased through the synthesis of three tick-derived thrombin-inhibiting proteins, each of which were assembled, purified and isolated for biological assays within a few hours.

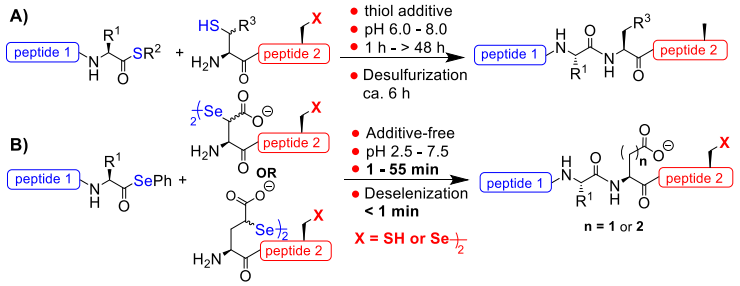
The Bigger Picture

Over the past decade there has been a renaissance in the use of large polypeptides and proteins as therapeutic agents meaning that there is significant need for technologies to rapidly and efficiently access these biomolecules. While biological expression systems serve a critical role in the production of polypeptides, these are not a 'one-stop shop'; some targets cannot be produced using recombinant methods due to peptide/protein toxicity, and the techniques are often limited to the incorporation of the 20 proteinogenic amino acids. The advent of peptide ligation technologies, based around the native chemical ligation methodology, have revolutionized protein science by providing an avenue to access polypeptides and proteins with tailor-made modifications to maximize specificity and activity or to probe biological function (e.g. through incorporation of post-translational modifications and fluorophores, respectively). One of the key problems remaining in the field is that often the time required to assemble a particular protein target is unacceptably long. In this article, we describe the development of a one-pot ligation–deselenization technology at aspartate and glutamate that enables the synthesis of native polypeptides and proteins on unprecedented timescales. The key feature of

the methodology is that both the ligation and deselenization reactions are chemoselective, proceed rapidly and cleanly, and are compatible for use in a one-pot regime. The power of the chemoselective deselenization step is highlighted through the synthesis of selenoprotein K, containing a native selenocysteine residue. Moreover, the utility of the technology is showcased through the synthesis of three small protein targets, which could be assembled and purified within a few hours. The methodology described here should serve as a powerful means to access synthetic proteins, including therapeutic leads, in the future.

INTRODUCTION

Native chemical ligation has revolutionized the field of protein science by facilitating access to native, modified and designer biomolecules for interrogative studies on structure and/or function.[1,2] The need for a cysteine residue (the least abundant proteinogenic amino acid) on the N-terminus of one of the reacting peptide fragments has motivated the development of β-, γ- and δ-thiolated variants of other amino acids[3-14] as well as thiol-containing auxiliaries that can be employed as Cys surrogates in ligation chemistry.[15-20] Following ligation reactions at these residues, the thiol auxiliary is desulfurized (usually by means of radical-based protocols[21]) in order to obtain native polypeptide products (Scheme 1A). However, this transformation is not chemoselective in the presence of other unprotected cysteine residues that might be found elsewhere in the sequence. This limitation of desulfurization chemistry has led to expansion of the native chemical ligation transformation to peptides bearing the 21st amino acid selenocysteine (Sec)[22-24] as well as selenoamino acids (specifically Pro[25] and Phe[26] to date). The key advantage of carrying out ligation chemistry at 'selenoamino acids' rather than 'thioamino acids' is that chemoselective deselenization can be performed under mild conditions (typically a phosphine reductant and a hydrogen-atom source) that do not affect unprotected Cys residues.[22] It has also been demonstrated independently by us[27] and Metanis and co-workers[28] that ligation products can be subjected to oxidative deselenization to afford Ser in place of Sec at the ligation junction.



Scheme 1. A) Native chemical ligation–desulfurization; B) One-pot additive–free diselenide–selenoester ligation–deselenization reaction at (β-Se)-Asp and (γ-Se)-Glu reported here. NB: when X = Se, Sec often exists as an intramolecular diselenide with (β-Se)-Asp and (γ-Se)-Glu before deselenization.

While there are clear benefits to using Sec or other selenoamino acids in native chemical ligation, the rates of these reactions in the presence of an aryl thiol as a reductant are generally slower than would be anticipated based on the significantly enhanced nucleophilicity of selenolates (compared with the corresponding thiolates used in native chemical ligation). These slow reaction rates can be rationalized by the low redox potential of Sec (–381 mV),[29] coupled with the weak reductive power of aryl thiols, which provides a low steady-state concentration of selenolate during the

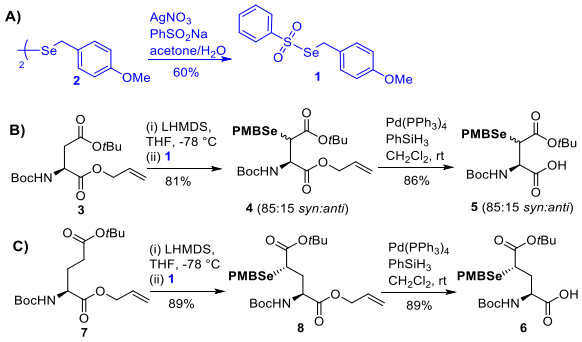
ligation reaction.[23] One solution is to employ stronger reducing agents, e.g., a phosphine. However, this promotes homolysis of the weak C–Se bond of Sec, a transformation that has been exploited for the chemoselective deselenization of Sec to Ala in the presence of free Cys (*vide supra*).[22,30] Metanis and co-workers have recently reported a workable solution to this deleterious side reaction at Sec *via* the use of TCEP in the presence of ascorbate as a radical trap.[31] However, this approach prevents *in situ* deselenization chemistry without prior removal of ascorbate from the reaction mixture.

We have recently reported that peptides possessing an N-terminal selenocystine moiety (the oxidized form of Sec) can be ligated to peptides bearing a C-terminal aryl selenoester in aqueous buffer without the use of any additives in the reaction.[32] These ligation reactions were demonstrated to have unparalleled reaction rates (1 – 10 min) compared with the corresponding thiol-mediated ligation reactions (1 h – > 48 h) and could be coupled with *in situ* radical deselenization (through the addition of TCEP and DTT) to afford native peptide products in excellent yields. Whilst this new additive–free methodology provides a significant advance in ligation technology, a synthetic bottleneck still exists in the deselenization step, which typically requires 6-16 h to reach completion. Clearly, enhancing the rate of the deselenization step would raise the possibility of generating native polypeptides and proteins on unprecedented timescales. Herein we demonstrate that peptides possessing N-terminal β-selenoaspartate [(β-Se)-Asp] or γ-selenoglutamate [(γ-Se)-Glu] residues can facilitate rapid and efficient additive–free ligation reactions with peptide selenoesters (Scheme 1B). Crucially, these ligations can be coupled with one-pot deselenization reactions that proceed cleanly in under a minute. The rapid nature of both the additive–free ligation and deselenization reactions at Asp and Glu enables the preparation of proteins in minutes, a feature which we highlight through the synthesis of three tick-derived protein thrombin inhibitors.

RESULTS

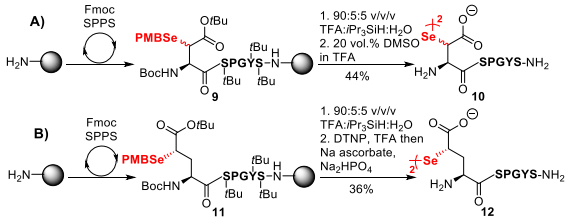
Synthesis of β-selenoaspartate and γ-selenoglutamate building blocks and incorporation into peptides

The synthesis of a suitably protected (β-Se)-Asp building block began with electrophilic selenylation chemistry, analogous to the sulfenylation transformation we recently reported in the synthesis of thioamino acids.[4,13,14] Initially, our intention was to incorporate a 2,4,6-trimethoxybenzyl (Tmb)-protected selenol unit into the target amino acid. However, the instability of the Tmb-protected selenosulfonate precluded isolation. We next investigated the preparation of a less electron-rich selenylating reagent, namely, *p*-methoxybenzyl (PMB) selenosulfonate **1**. This reagent was prepared by treatment of diselenide **2** with $AgNO_3$ in the presence of sodium benzenesulfinate (Scheme 2A and Figures S103-S106). Selenylation was next effected through the addition of selenosulfonate **1** to the dianion of orthogonally-protected Asp **3** at low temperature and provided β-Se amino acid **4** in 81% yield as an 85:15 *(syn:anti)* mixture of diastereomers (inseparable by flash column chromatography but separable by C18 reverse-phase HPLC, *vide infra*, Scheme 2B). Finally, allyl ester deprotection was facilitated by treatment with (tetrakis)triphenylphosphine palladium (0) and phenylsilane to afford the desired building block **5** (85:15 *syn:anti*) in excellent yield (Figures S107-S110). Similarly, suitably protected (γ-Se)-Glu building block **6** could be prepared by selenylation of Boc-Glu(O*t*Bu)-OAll **7** followed by allyl ester deprotection in excellent yield (Scheme 2C). On this occasion **6** was prepared as a single diastereoisomer (2*S*, 4*S* as determined by NMR spectroscopy) due to the exquisite stereoselectivity of the selenylation reaction on the Glu substrate (Figures S111-S114).



Scheme 2. Synthesis of A) selenylating reagent 1; B) PMB-protected β-selenyl aspartate 5, and C) PMB-protected γ-selenyl glutamate 6

The protected selenylated aspartate building block **5** was next incorporated into the N-terminus of a model peptide using Fmoc-strategy SPPS to afford resin-bound **9** (see Scheme 3A and Supplemental Information). Following acidolytic side-chain deprotection/cleavage from the resin, oxidative deprotection of the PMB selenoether protecting group (20% DMSO in TFA) and purification by reverse-phase HPLC, the peptide diselenide dimer **10** was isolated in 44% yield based on the original resin loading. It should be noted that the acidity of the β-proton of **5** led to epimerization during coupling to afford a 1:1 (*syn:anti*) mixture of diastereomers of the final peptide product **10** (Figures S1 and S2). This was inconsequential to the purity of the final product as the stereocenter is destined for removal through deselenization after the ligation event (Figure S3). We also demonstrated that the nature of the stereochemistry at the β-center does not lead to significant differences in ligation rate. Specifically, when HPLC-separated *syn* and *anti* diastereomers of **4** (Scheme 2B) were deprotected and subjected to an additive–free diselenide–selenoester ligation reaction, both reached completion at similar time points (see Supplemental Information for details). Selenylated glutamate **6** could also be coupled to the N-terminus of a resin-bound peptide to afford **11** followed by acidolytic deprotection and cleavage from the resin (Scheme 3B). On this occasion PMB-deprotection was best effected by DTNP (5.2 eq.) in TFA to afford the corresponding selenyl sulfide which could be removed by treatment with a solution of 40 mM ascorbate in phosphate buffer to provide the desired peptide target in 36% yield based on the original resin loading (Figure S4). In contrast to selenopeptide **10**, **12** was produced as a single diastereomer (Figure S5).



Scheme 3. Synthesis of model peptides A) 10 bearing an N-terminal (β-Se)-Asp and B) 12 bearing an N-terminal (γ-Se)-Glu.

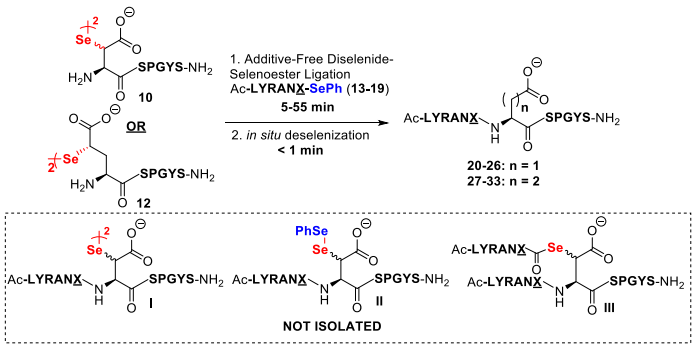
One-pot additive–free diselenide–selenoester ligation–deselenization reactions at (β-Se)-Asp and (γ-Se)-Glu

With model diselenide dimer peptides **10** and **12** in hand, we next explored additive–free ligation reactions with a range of model selenoesters (**13–19**). Specifically, **10** or **12** and a given peptide selenoester (see Supplementary

Information for selenoester synthesis and Figures S6-S12 for characterization data) were simply dissolved in 6 M Gdn•HCl, 0.1 M phosphate buffer, at a final pH of 6.2–6.5 (with no additives or pH adjustment necessary). Gratifyingly, all reactions proceeded cleanly to afford the desired ligation products in excellent yield as judged by UPLC-MS analysis. As reported in our prior investigations on the selenocystine–selenoester ligation,[32] multiple products are observed from the ligation reaction (denoted as **I**–**III** for ligation at (β-Se)-Asp in Table 1). While symmetric diselenide **I** is usually observed as the major product, together with a small amount (<10%) of asymmetric diselenide product **II**, product selenoester **III** is observed when an excess of the selenoester fragment is used in the reaction and/or when selenoesters bearing sterically hindered C-terminal residues, e.g. Val and Leu, are employed. However, the ratio of these products is inconsequential for the overall efficiency of the reaction as these converge into the single native polypeptide product following deselenization. The rates of the additive–free diselenide–selenoester ligation reactions at both (β-Se)-Asp and (γ-Se)-Glu were rapid, with all reactions reaching completion between 5 and 55 min [as judged by UPLC-MS analysis and by precipitation of diphenyldiselenide (DPDS) – a visual prompt for completion of the reactions]. Notably, ligations at selenoesters bearing C-terminal Ala (**13**), Ser (**14**), Phe (**15**), Tyr (**16**), Met (**17**) and Leu (**18**) were complete between 5–15 min, significantly faster than native chemical ligation at Cys and the homologous β-mercapto-Asp[4] and γ-mercapto-Glu.[14] Furthermore, ligation with selenoester **19** with a sterically hindered C-terminal Val residue was complete in 55 minutes at Asp and 45 min at Glu, again significantly faster than the thiolate equivalents (β-mercapto-Asp[4] and γ-mercapto-Glu[14]; 16 h).

Upon completion of the additive–free ligations, crude reaction mixtures were subjected to *in situ* deselenization. This involved extraction of the precipitated DPDS with hexane, followed by treatment with TCEP (50 eq.) and DTT (5 eq.). Remarkably, the deselenization of (β-Se)-Asp and (γ-Se)-Glu proceeded cleanly and to completion (in all cases) within 60 seconds, cf. deselenization at Sec that requires 4–6 hours (see Figures S13-S40 for data). Interestingly, the deselenization was also complete within a minute at a wide (1.8–7.0) pH range (see Figures S54–S58). The resulting products from the one-pot additive–free diselenide–selenoester ligation–deselenization reactions were subsequently purified by reverse–phase HPLC to afford the native peptide products **20**–**26** (for ligations at (β-Se)-Asp) and **27**–**33** for ligations at (γ-Se)-Glu in excellent yield (52–91%) over the two steps (Table 1 and Figures S13-S40). Importantly, the exceptional rates of the additive–free ligation and deselenization reactions at (β-Se)-Asp and (γ-Se)-Glu enable access to native peptides in minutes (including at sterically hindered junctions), a timescale that, to our knowledge, cannot be achieved with currently available techniques.

Table 1. Scope of the one–pot additive–free diselenide–selenoester ligation–deselenization at (β-Se)-Asp and (γ-Se)-Glu.



Entry	Peptide diselenide	Selenoester (X)	Ligation Time (min)	Yield for One-Pot Ligation–Deselenization
1	**10**	**13:** X = A	5 [a]	**20:** 64%
2	**10**	**14:** X = S	10 [a]	**21:** 57%
3	**10**	**15:** X = F	10 [a]	**22:** 53%
4	**10**	**16:** X = Y	5 [a]	**23:** 64%
5	**10**	**17:** X = M	5 [a]	**24:** 65%
6	**10**	**18:** X = L	15 [a]	**25:** 72%
7	**10**	**19:** X = V	55 [b]	**26:** 58%
8	**12**	**13:** X = A	10 [a]	**27:** 77%
9	**12**	**14:** X = S	10 [a]	**28:** 76%
10	**12**	**15:** X = F	10 [a]	**29:** 91%
11	**12**	**16:** X = Y	15 [a]	**30:** 60%
12	**12**	**17:** X = M	15 [a]	**31:** 67%
13	**12**	**18:** X = L	10 [a]	**32:** 67%
14	**12**	**19:** X = V	45 [b]	**33:** 52%

Conditions: *Additive-free ligation;* 2.5 mM final concentration of diselenide dimer in 6 M Gdn•HCl, 0.1 M Na_2HPO_4, pH 7.2 (reduces to 6.2-6.5 upon addition to peptide fragments). *One–pot deselenization;* hexane extraction (x5) followed by addition of 0.25 M TCEP, 25 mM DTT in 6 M Gdn•HCl, 0.1 M Na_2HPO_4, pH 5 – 6.

[a] 0.5 eq. of H-(β-Se)DSPGYS-NH_2 dimer or H-(γ-Se)ESPGYS-NH_2 dimer to 1.3 eq. of selenoester.

[b] 0.5 eq. of H-(β-Se)DSPGYS-NH_2 dimer or H-(γ-Se)ESPGYS-NH_2 dimer to 2.0 eq. of selenoester.

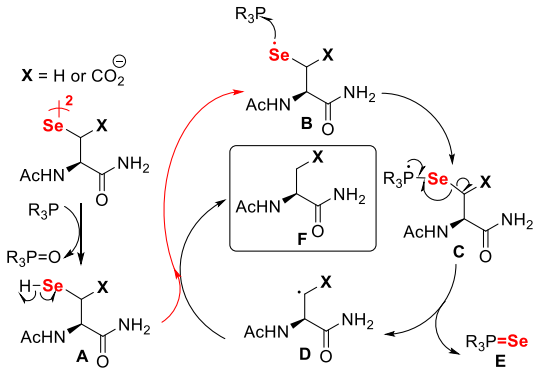
NB: products from the additive-free ligation at β-Se-Asp **I-III** (not isolated) shown in box.

Ligation–deselenization using additives

Very recently it has been reported that native chemical ligation reactions at Sec (using peptide thioesters as the acyl donor) can be performed in the presence of the reductant TCEP (that normally facilitates deselenization) through the addition of ascorbic acid.[31] We were therefore interested to assess whether (β-Se)-Asp could be employed in "additive ligations" through a native chemical ligation pathway. To this end, we reacted **10** with peptide selenoester **19** in the presence of 50 mM TCEP and 100 mM ascorbic acid in ligation buffer.[31] Unfortunately, these conditions resulted in complete deselenization of starting diselenide dimer peptide **10** with no detectable ligation product (Figures S41 and S42). Given that these conditions prevent deselenization of Sec-containing peptides (see[31] and Figures S43 and S44), this observation reflects the increased lability of the C–Se bond in the β-Se Asp moiety (*vide supra*). We therefore sought to optimize additive conditions using an alternative radical trap, namely diphenyldiselenide (DPDS), in place of ascorbic acid. Optimized conditions involved ligation between **10** and **19** at a saturating concentration of DPDS (250 mM) and 25 mM TCEP (Figures S45-47). While a significant amount of the desired product was formed (in deselenized form), deselenization of starting peptide **10** could not be prevented entirely, thus lowering the overall yield of the reaction.

Mechanistic insight into the rapid deselenization reaction

Having established that ligation reactions at (β-Se)-Asp and (γ-Se)-Glu must be strictly performed under an additive–free regime, we moved to further explore the exceptional rate enhancement observed for deselenization at these amino acids compared with Sec (see Supplemental Information). The current mechanistic model for deselenization using a phosphine, e.g. TCEP, and a hydrogen-atom source, e.g. DTT, invokes an initial reduction of the diselenide (or selenyl sulfide for native chemical ligation) to afford a selenol (see **A**, Scheme 4), which could serve as a precursor to a small amount of Se-centered radical **B**.[22,28] It is also feasible that **B** could be generated from the starting diselenide.[33] Regardless of the pathway to the selenium-centered radical, **B** could react rapidly with the phosphine to generate the phosphorus-centered radical species **C**. C–Se bond homolysis of **C** would generate a β-carbon-centered radical **D** and phosphine selenide **E** (proposed to be a key driving force for the reaction). Hydrogen-atom abstraction by the β-carbon centered radical **D** could then produce the native amino acid **F**. Interestingly, we have demonstrated that deselenization reactions proceed to completion (and at similar rates) at (β-Se)-Asp even in the absence of an H-atom source such as DTT (Figure S59). This suggests that the H-atom abstraction may be possible from selenol **A** (produced by TCEP reduction of the starting diselenide) that would regenerate **B** and propagate a radical chain as depicted in Scheme 4.



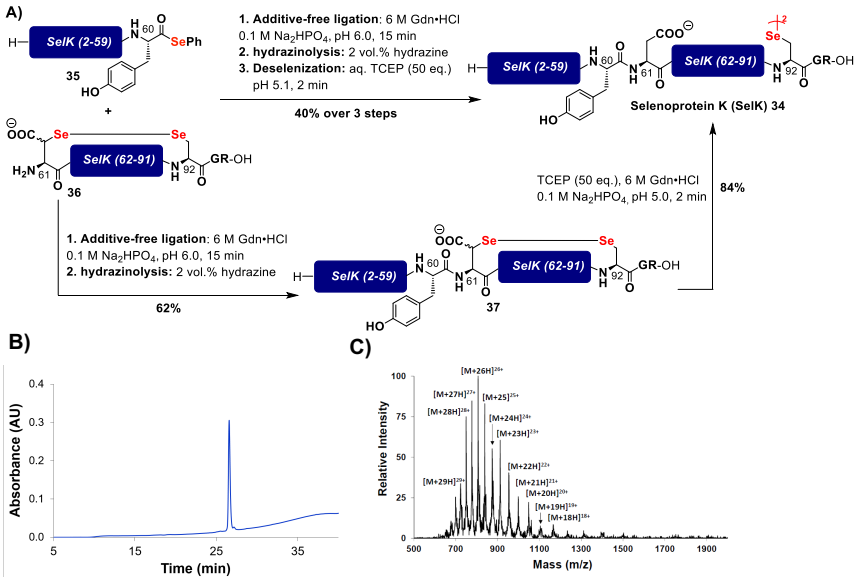
Scheme 4. Putative pathways for the deselenization of selenoamino acids. For TCEP, R = $CH_2CH_2CO_2^-$

To help understand the significant rate enhancement observed for deselenization, initially at (β-Se)-Asp, we probed the energies associated with the formation of the proposed intermediates with computational quantum chemistry calculations. These were performed with the Gaussian 09 program,[34] using the species depicted in Scheme 4 as models (Table S1). Gas-phase energies were obtained at the DSD-PBEP86/aug′-cc-pVTZ level,[35] with the effect of solvation incorporated through the SMD continuum model at the M05-2X/6-31G(d) level. Our calculated energies corresponding to the individual steps for the pathways shown in Scheme 4, together with a schematic energy profile are included in the Supplemental Information (Tables S1 and S2 and Figure S115). Thus, the barrier calculated for **C** → **D** for the unsubstituted system (X = H) is 23.4 kJ mol^{-1} whereas that for the aspartate derivative (X = CO_2^-) is –5.3 kJ mol^{-1}, the negative value indicating that this modification essentially removes the barrier (Table S1 and Figure S115). This is owing to stabilization of the electron-deficient radical center by the anionic β-carboxylate moiety. Corresponding calculated spin densities for species involved in the conversion of **C** to **D** are consistent with the energy data (Figure S116). In addition, abstraction of the H atom from DTT by carbon-centered radical **D** is predicted to have a lower barrier of 5.3 kJ mol^{-1} for (β-Se)-Asp (X = CO_2^-) compared with Sec (X = H, 10.9 kJ mol^{-1}). We performed the analogous computational experiments for the

proposed pathway in Scheme 4 with (γ-Se)-Glu (Tables S1 and S2 and Figure S115). Perhaps unsurprisingly, the key barrier for cleavage of the C–Se bond in TCEP adduct **C** to afford carbon-centered radical **D** was very similar to that calculated for the Asp homologue (Table S1 and Figure S115), which agrees with our experimental observation, i.e. that deselenization at both (β-Se)-Asp and (γ-Se)-Glu are rapid.

Selectivity of deselenization

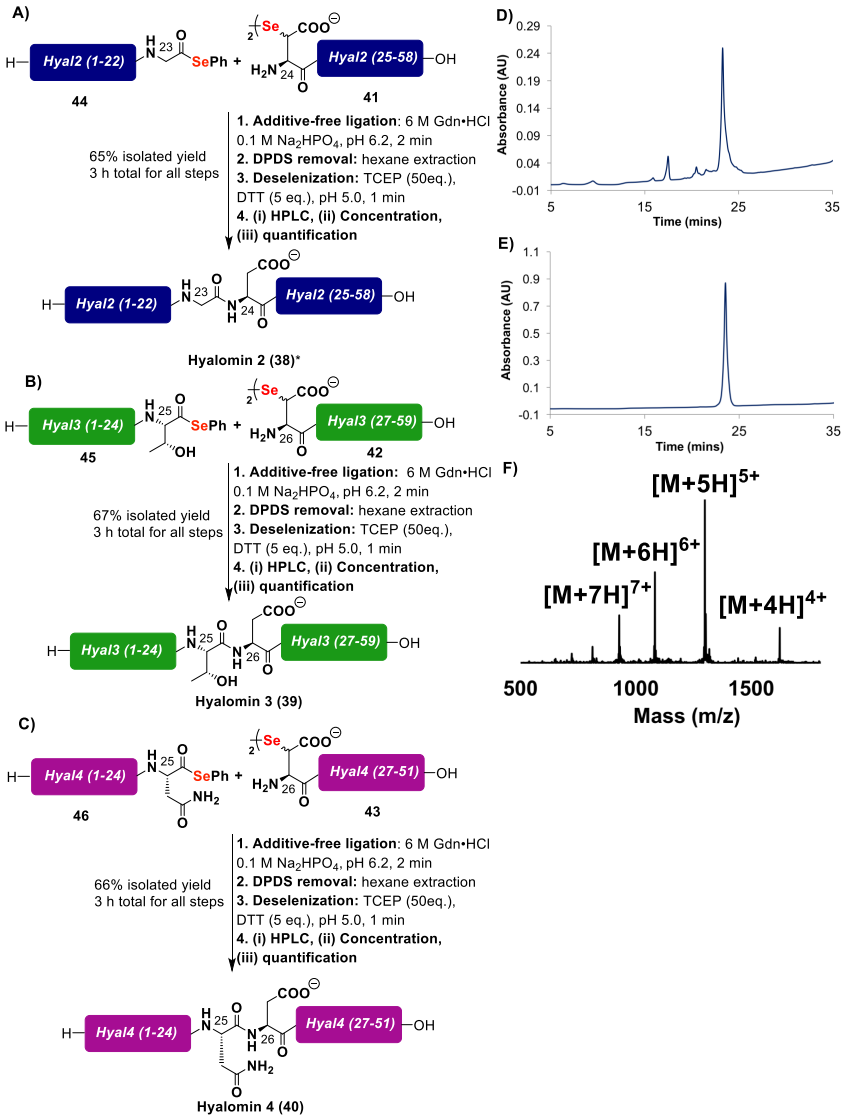
Given the expedient nature of the deselenization step, we were interested in probing whether selective deselenization of (β-Se)-Asp and (γ-Se)-Glu would be possible without concomitant conversion of Sec to Ala. Towards this end, we first demonstrated that (β-Se)-Asp and (γ-Se)-Glu could be selectively deselenized in a model peptide that also possessed an unprotected Sec (Figures S48–S53). Having demonstrated the chemoselectivity on a model system, we next embarked on the assembly of a protein target that would benefit from the chemoselective deselenization transformation. For this purpose, we chose the 93 amino acid protein selenoprotein K (SelK) **34** (residues 2-94), possessing a Sec residue at position 92 that cannot be used to assemble the protein *via* ligation due to its C-terminal proximity. Unlike most selenoproteins that possess selenosulfide linkages, SelK exists as a homodimer linked *via* an intermolecular diselenide at Sec92.[36] While the exact biological role of SelK has not been established, Sec92 and the high redox potential of the intermolecular diselenide bond is thought to be important for protein function.[37] Assembly of peptide selenoester **35** and peptide **36** bearing an N-terminal (β-Se)-Asp and an internal Sec residue (linked as an intramolecular diselenide) was first performed using Fmoc-SPPS methods (Scheme 5, Supplementary Information and Figures S60-S63). Peptide **36** was prepared with two norleucines substituted for methionine (Met) residues owing to significant but incomplete oxidation of the thioether side chains of Met during the acidic deprotection and cleavage conditions of the peptide fragments that complicated analysis. Ligation under the additive–free conditions was performed by simply dissolving the two fragments in aqueous ligation buffer (adjusted to pH 6.0) using a two-fold excess of selenoester **35**. Following 15 min the reaction had proceeded to completion to afford a mixture of intramolecular diselenide **37** and the selenoester-linked ligation product, as judged by UPLC-MS analysis. Following hydrazinolysis of the unproductive selenoesters, **37** was afforded as the exclusive ligation product (Figure S64). The intramolecular diselenide ligation product **37** was subsequently isolated in 62% yield following reverse-phase HPLC (Figures S65 and S66). Gratifyingly, treatment of **37** with TCEP, in the absence of DTT, for 2 min led to chemoselective deselenization of the (β-Se)-Asp without any observed Sec deselenization. Purification subsequently afforded SelK **34** in 84% yield (Figure S67). Mass spectrometric analysis confirmed that **34** was isolated as the homodimer with a molecular weight of 21 kDa (Figure S68).[38] Having successfully showcased the chemoselective deselenization in the synthesis of SelK with intermediary purification, we next attempted to rapidly access the selenoprotein using a one-pot protocol (Scheme 5A). Towards this end, rapid additive–free ligation between **35** and **36**, followed by *in situ* hydrazine treatment and chemoselective deselenization *via* treatment with TCEP provided SelK together with the acyl hydrazide of selenoester fragment **35** (Figure S69). Following reverse-phase HPLC purification, homodimeric SelK **34** was isolated in 40% yield and in excellent purity over the three synthetic steps (Scheme 5B and 5C and Figures S70 and S71).



Scheme 5. A) Synthesis of homodimeric SelK *via* additive–free diselenide–selenoester ligation at (β-Se)-Asp followed by chemoselective deselenization in the presence of native and unprotected Sec92 ; B) HPLC trace of purified synthetic SelK following one–pot ligation–deselenization protocol, rt = 26.6 min, λ = 220 nm; C) ESI mass spectrum of synthetic SelK homodimer 34.

One-pot synthesis of the hyalomins *via* ligation–deselenization

In our previously described selenocystine–selenoester ligation,[32] the deselenization of Sec was the synthetic bottleneck, requiring 4–6 h to reach completion. As such, this step prevented full exploitation of the exceptional rate of the additive–free ligation reaction. We envisaged that the increase in deselenization rate at (β-Se)-Asp, coupled with the fast additive–free ligation rates at this residue, would provide a unique means to access target proteins by chemical synthesis within hours rather than days, a feature that was showcased in the synthesis of SelK. To investigate this possibility further, we sought to prepare a selection of small thrombin-inhibiting hyalomin proteins (hyalomin-2, hyalomin-3 and hyalomin-4) using our methodology. The hyalomins are a family of four cysteine-free proteins produced within the salivary glands of the tick *Hyalomma marginatum rufipes* that support the blood-feeding activity of the organism.[39] The absence of Ala residues at a site that would permit assembly through other ligation approaches, together with the wealth of acidic residues within the sequences (pI < 4), made these amenable to assembly using our ligation technology. Disconnection of hyalomins 2–4 (**38**–**40**) was made at Asp residues close to the middle of the sequences. The C-terminal diselenide dimer peptide fragments **41**–**43** were synthesized on 2-chlorotrityl chloride resin via Fmoc-SPPS with the incorporation of (β-Se)-Asp building block **5** at the N-terminus (Figures S72-S77). N-terminal peptides were also prepared on 2-chlorotrityl chloride resin via Fmoc-SPPS and converted to C-terminal phenylselenoesters **44**–**46** (Figures S78-S83).



Scheme 6. Synthesis of thrombin-inhibiting proteins A) hyalomin-2, B) hyalomin-3 and C) hyalomin-4 *via* one-pot additive-free diselenide–selenoester ligation at (β-Se)-Asp. All products were synthesized, purified, characterized and quantified within a 3 h time period; D) crude HPLC of one-pot ligation–deselenization to afford hyalomin-2 (38), rt = 23.5 min, λ = 280 nm; E) HPLC of purified hyalomin-2 (38), rt = 23.5 min, λ = 280 nm; F) ESI-mass spectrum of hyalomin-2 (38). * Hyalomin-2 was produced with Met18 oxidized. The sulfoxide could be reduced in a subsequent step to afford the native protein in 96% yield (see Supplemental Information for details).

With the requisite fragments in hand, we next set out to prepare each of the hyalomin targets with an emphasis on synthesis, purification and isolation within a short timeframe. Towards this end, diselenide dimer fragments **41**–**43** (1 eq. based on the monomeric peptide) were reacted with peptide selenoesters **44**–**46** (1.5 eq. and 1.6 eq. for **44** and **46** respectively and 2 eq. for **45**) in ligation buffer (pH adjusted to 6.2) under additive–free conditions (Scheme 6). Gratifyingly, the reactions proceeded in just 2 mins to afford the desired ligation products as a mixture of the symmetrical diselenide dimer and product bearing a selenoester linkage (as judged by UPLC-MS analysis). Subsequent deselenization was effected via addition of TCEP (50 eq.) and

DTT (5 eq.) and, like the model systems, proceeded smoothly and to completion within 1 min (Figure S84, S89 and S94). It should be noted that the DTT was added to the reaction to thiolyze the product selenoester to the corresponding selenol, which could be subsequently deselenized with TCEP. The crude hyalomins were purified by RP-HPLC over a 30 min gradient, and fractions containing the desired protein were analysed using a UPLC-MS system with a gradient of 3 min, thus allowing numerous samples to be run over a short period. The relevant fractions were pooled and the solvent removed on a Genevac solvent evaporation system over 1 h at 50 °C and 3 mbar. The pure proteins were re-dissolved in distilled water and the concentration confirmed by NanoDrop UV-Vis to determine concentrations of the protein solutions (for direct use in thrombin inhibitory assays). Following the one–pot diselenide–selenoester ligation–deselenization and purification, the hyalomins were isolated in excellent yields (65–67%). Importantly, following ligation of the peptide fragments, purification, characterization, solvent removal and quantification of the pure proteins, each of the hyalomins were generated within an impressively brief 3 h period in >98% purity (Figures S84-S98). Finally, having accessed hyalomins 2–4 (**38–40**) we next assessed the activity of the synthetic proteins as inhibitors of human thrombin (see Supplemental Information for details). Both hyalomin 2 (**38**) and hyalomin 3 (**39**) proved to be extremely potent thrombin inhibitors with inhibition constants (K_i) of 1.24 ± 0.05 nM and 14.73 ± 0.64 nM, respectively (Figures S99-S102). Surprisingly, hyalomin-4 (**40**) exhibited weaker inhibitory activity (IC_{50} = 20 μM), possibly owing to the significantly shorter C-terminal tail region of this protein compared with **38** and **39**. Future work in our laboratories will focus on determining the three-dimensional structure of thrombin-hyalomin complexes to elucidate their inhibitory binding mode.

Conclusions

In summary, we have developed a short and efficient synthesis of suitably protected (β-Se)-Asp and (γ-Se)-Glu building blocks. We have demonstrated that these selenylated amino acids can be incorporated into resin-bound peptides and facilitate rapid, additive–free ligation reactions with peptide selenoesters. Following the ligation event, and without purification, deselenization of the β- and γ-seleno auxiliaries was smoothly effected within 1 min in all cases. The impressive rates of deselenization at (β-Se)-Asp were highlighted in the synthesis of SelK, whereby the β-seleno auxiliary on Asp could be chemoselectively deselenized in the presence of Sec to afford the native selenoprotein. Furthermore, the rapid rates of both the ligation and deselenization steps were showcased in the one–pot assembly of three thrombin inhibitory proteins from the hyalomin family, which could all be assembled, purified and isolated within 3 h for immediate assessment in thrombin inhibition assays. The simplicity, efficiency and speed of the ligation–deselenization chemistry described here should see the technology applied to the synthesis of numerous protein targets and protein libraries.

EXPERIMENTAL PROCEDURES

General procedure for one-pot additive–free diselenide–selenoester ligation–deselenization

The diselenide dimer peptide **10** [H-(β-Se)DSPGYS-NH_2] (2.0 mg, 1.4 μmol) or **12** [H-(γ-Se)ESPGYS-NH_2] (1.0 mg, 0.7 μmol) and a selenoester Ac-LYRANX-SePh (**13-19**) (3.6 μmol for reaction with **10** or 1.8 μmol for reaction with **12**) were separately

dissolved in ligation buffer (6 M Gdn•HCl, 0.1 M Na_2HPO_4, pH = 7.1) to a

concentration of 10 mM (with respect to the selenopeptide fragment) and 13 mM (with respect to the selenoester fragment or 20 mM in the examples with Ac-LYRANV-SePh). The selenoester solution was added in one portion to the solution of diselenide in an Eppendorf tube and the reaction mixture left at rt with intermittent agitation. Analytical HPLC-MS analysis indicated consumption of the diselenide and formation of ligation products at the times indicated in Table 1. The ligation reaction mixture was washed with an equal volume of hexane (×5) to remove diphenyldiselenide (DPDS) and sparged with nitrogen for 5–10 min. Separately a solution of TCEP (0.25 M) and DTT (25 mM) was prepared in ligation buffer and the pH adjusted to 5 – 6. An equal volume of the TCEP/DTT solution was added in one portion to the ligation reaction mixture and the reaction left for 5 min (after 1 min HPLC-MS analysis indicated that the ligation products had already been consumed and the deselenized peptide had been formed). The reaction mixture was diluted with water containing 0.1% TFA, purified by preparative HPLC and lyophilized to give the native peptide product as a white solid (see Supplemental Information for UPLC data of crude reactions and characterization data of purified peptide products).

SUPPLEMENTAL INFORMATION

Supplemental Information includes experimental procedures, crude reaction traces and characterization data for all novel compounds and thrombin inhibitory data. The file includes 101 supplemental figures and geometries for computational studies.

AUTHOR CONTRIBUTIONS

Conceptualization, P. J. B. P., L. R., and R. J. P. Methodology, N. J. M., J. S., S. S. K., D. C., A. M. G., J. R-R., B. C., and R. J. P.; Investigation, N. J. M., J. S., S. S. K., D. C., A. M. G., J. R-R. and B. C.,; Writing – Original Draft, N. J. M., L. R., and R. J. P. Writing – Review & Editing, N. J. M., J. S., S. S. K., D. C., A. M. G., J. R-R., B. C., P. J. B. P., L. R., and R. J. P. Funding Acquisition, P. J. B. P., L. R., and R. J. P.; Supervision, P. J. B. P., L. R., and R. J. P.

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