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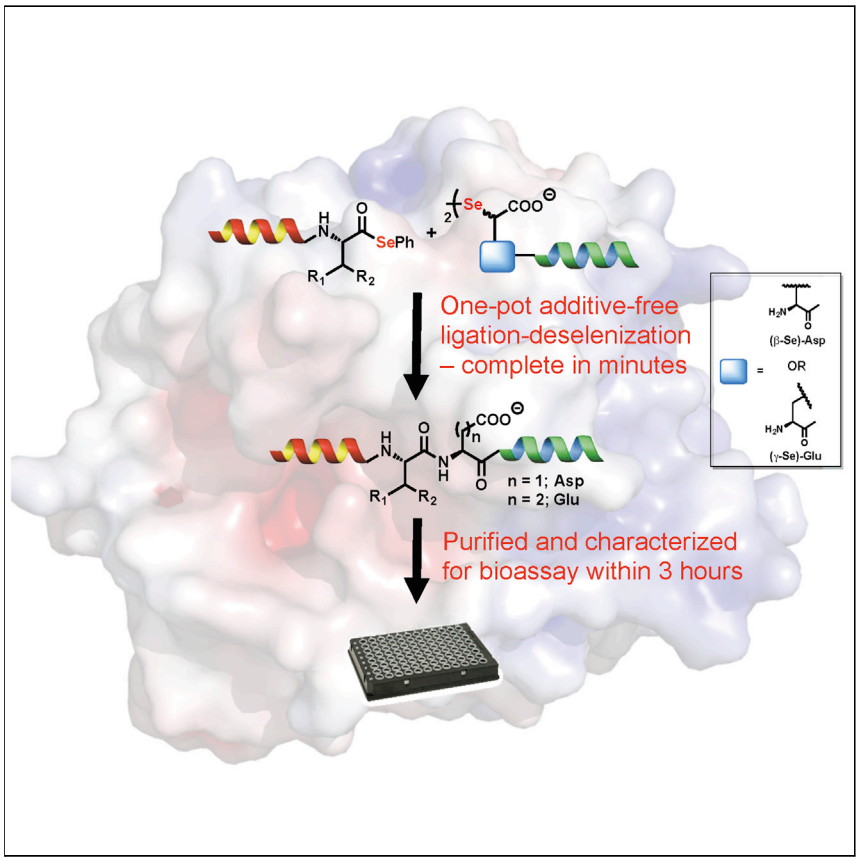
Accelerated Protein Synthesis via One-Pot Ligation-Deselenization Chemistry



Here, we describe the development of a highly efficient one-pot ligation-deselenization technology at aspartate and glutamate that enables the synthesis of polypeptides and proteins on unprecedented timescales. The power of the methodology is showcased through the rapid assembly of three thrombin-inhibiting tick-derived proteins as well as the synthesis of the 21 kDa homodimeric selenoprotein K. This work lays the foundation for the facile synthesis of a range of bioactive polypeptides and proteins in the future.

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HIGHLIGHTS

Rapid one-pot ligation-deselenization at β-selenoaspartate and γ-selenoglutamate

Methodology enables chemical protein synthesis on unprecedented timescales

Synthesis of Selenoprotein K through chemoselective deselenization of β-selenoaspartate

Synthesis, purification, and quantification of thrombin inhibitory proteins in 3 hr

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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. Here, 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 timescales. 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 methodology described here should serve as a powerful means of accessing synthetic proteins, including therapeutic leads, in the future.

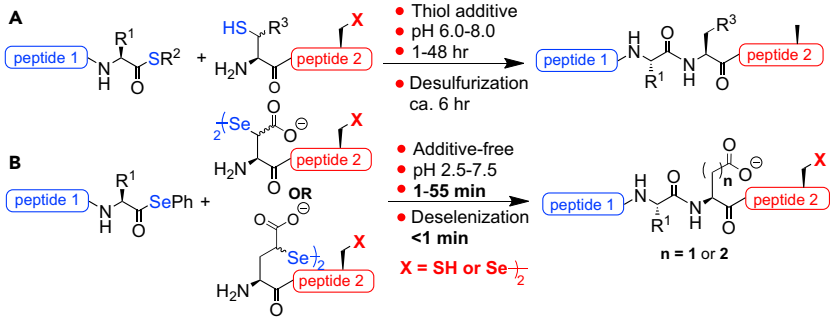
INTRODUCTION

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 used as Cys surrogates in ligation chemistry.[15-20] After ligation reactions at these residues, the thiol auxiliary is desulfurized (usually by means of radical-based protocols[21]) to yield 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 with a phosphine reductant and a hydrogen-atom source) that do not affect unprotected Cys residues.[22] We[27] and Dery et al.[28] have also independently demonstrated that ligation products can be subjected to oxidative deselenization to afford Ser in place of Sec at the ligation junction.

Although 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 given the significantly enhanced nucleophilicity of selenolates (in comparison with the corresponding

The Bigger Picture

A renaissance in the use of proteins as therapeutic agents has prompted the need for novel technologies to rapidly generate these biomolecules. The advent of peptide ligation technologies has revolutionized protein science by facilitating access to proteins with tailor-made modifications to maximize specificity and activity. However, one of the key problems remaining in the field is the unacceptably long time required for the assembly of protein targets. Here, we describe the development of a ligation-deselenization technology at aspartate and glutamate that enables the synthesis of native polypeptides and proteins on unprecedented timescales. Importantly, both the ligation and the deselenization reactions are chemoselective, proceed rapidly and cleanly, and are compatible for use in a one-pot regime, features that are showcased through the assembly of four small protein targets. The methodology should find use as a powerful means of rapidly accessing therapeutic proteins in the future.



Scheme 1. Ligation-Desulfurization and Ligation-Deselenization Methods

(A) Native chemical ligation-desulfurization.

(B) The one-pot additive-free diselenide-selenoester ligation-deselenization reaction at (β-Se)-Asp and (γ-Se)-Glu reported here. When X = Se, Sec often exists as an intramolecular diselenide with (β-Se)-Asp and (γ-Se)-Glu before deselenization.

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 use 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 (see above).[22,30] Reddy et al.[31] have recently reported a workable solution to this deleterious side reaction at Sec via the use of tris(2-carboxyethyl)phosphine (TCEP) in the presence of ascorbate as a radical trap. 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) in comparison with the corresponding thiol-mediated ligation reactions (1–48 hr) and could be coupled with in situ radical deselenization (through the addition of TCEP and DTT) to afford native peptide products in excellent yields. Although 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 hr to reach completion. Clearly, enhancing the rate of the deselenization step would raise the possibility of generating native polypeptides and proteins on unprecedented timescales. Here, 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 that 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

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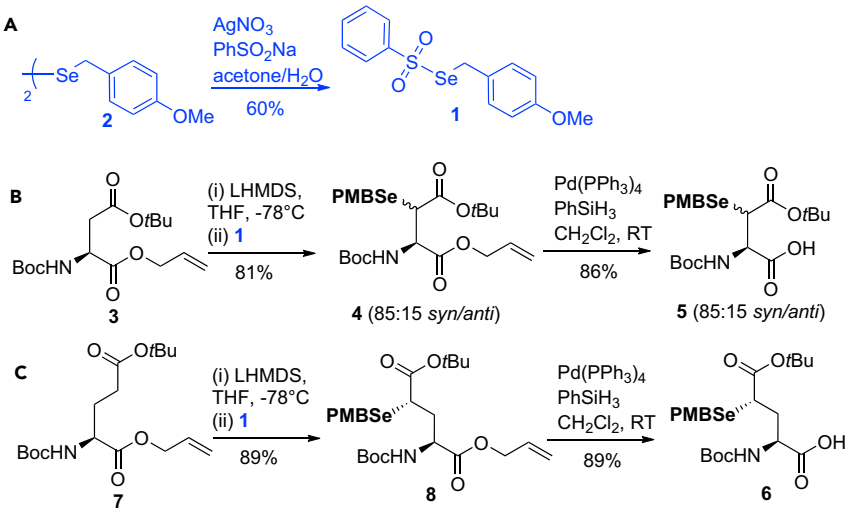
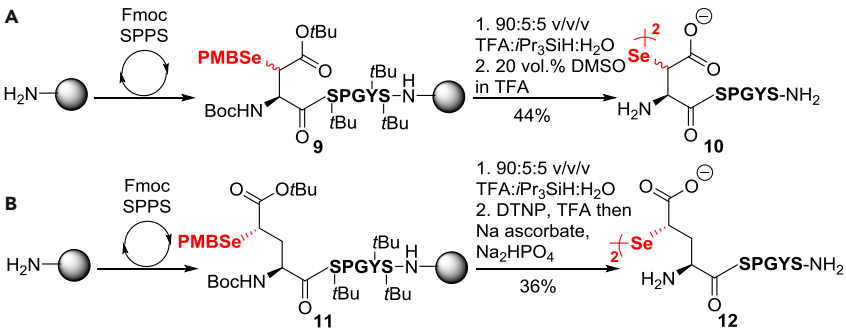


Scheme 2. Synthesis of β-Selenoaspartate and γ-Selenoglutamate Building Blocks
Synthesis of (A) selenylating reagent **1**, (B) PMB-protected β-selenyl aspartate **5**, and (C) PMB-protected γ-selenyl glutamate **6**.

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 high-performance liquid chromatography [HPLC]; see below and 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** and subsequent 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) as a result of the exquisite stereoselectivity of the selenylation reaction on the Glu substrate (Figures S111–S114).

The protected selenylated aspartate building block **5** was next incorporated into the N terminus of a model peptide by Fmoc-strategy solid-phase peptide synthesis (SPPS) to afford resin-bound **9** (see Scheme 3A and Supplemental Information). After acidolytic side-chain deprotection and cleavage from the resin, oxidative deprotection of the PMB selenoether-protecting group (20% DMSO in trifluoroacetic acid [TFA]), and purification by reverse-phase HPLC, the peptide diselenide dimer **10** was isolated in 44% yield according to the original resin loading. 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 because the stereocenter is destined for removal through deselenization after the ligation event (Figure S3).



Scheme 3. Synthesis of Model Peptides Incorporating N-Terminal Selenoamino Acids

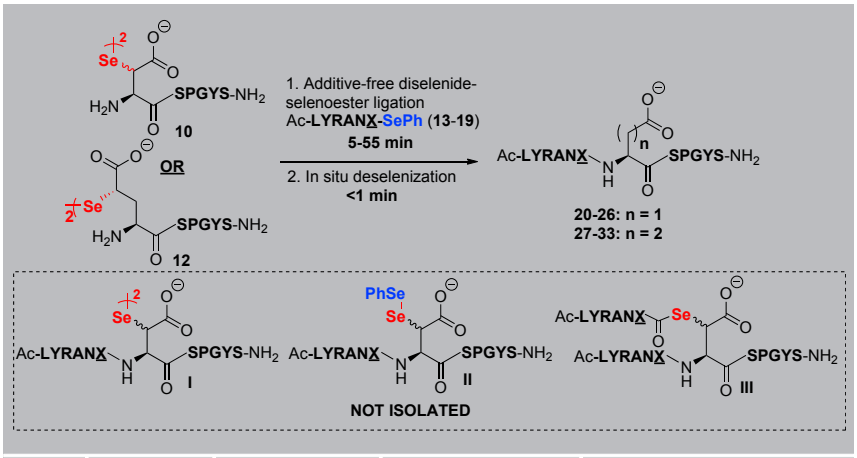
Synthesis of model peptides (A) **10** bearing an N-terminal (β-Se)-Asp and (B) **12** bearing an N-terminal (γ-Se)-Glu.

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**, which was subsequently subjected to acidolytic deprotection and cleavage from the resin (Scheme 3B). On this occasion, PMB deprotection was best effected by 2,2′-dithiobis(5-nitropyridine) (DTNP; 5.2 equiv) 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 according to the original resin loading (Figure S4). In contrast to selenopeptide **10**, **12** was produced as a single diastereomer (Figure S5).

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 Supplemental Experimental Procedures for selenoester synthesis and Figures S6–S12 for characterization data) were simply dissolved in 6 M Gdn•HCl and 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 ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) analysis. As reported in our previous investigations on 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). Whereas 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 used. However, the ratio of these products is inconsequential for the overall efficiency of the reaction because these converge into the single native polypeptide product after deselenization. The rates of the additive-free diselenide-selenoester ligation reactions at both (β-Se)-Asp and (γ-Se)-Glu were rapid, such that all reactions reached 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

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 of additive-free ligation: 2.5 mM final concentration of diselenide dimer in 6 M Gdn•HCl, and 0.1 M Na_2HPO_4 (pH 7.2; reduced to 6.2–6.5 upon addition to peptide fragments). Conditions of one-pot deselenization: hexane extraction (×5) followed by the addition of 0.25 M TCEP, 25 mM DTT in 6 M Gdn•HCl, and 0.1 M Na_2HPO_4 (pH 5–6). Products from the additive-free ligation at (β-Se)-Asp **I–III** (not isolated) are shown in the box.

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

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

selenoesters bearing C-terminal Ala (**13**), Ser (**14**), Phe (**15**), Tyr (**16**), Met (**17**), and Leu (**18**) were complete in 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 min at Asp and 45 min at Glu, again significantly faster than the thiolate equivalents (β-mercapto-Asp[4] and γ-mercapto-Glu[14]; 16 hr).

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 and subsequent treatment with TCEP (50 equiv) and DTT (5 equiv). Remarkably, the deselenization of (β-Se)-Asp and (γ-Se)-Glu proceeded cleanly

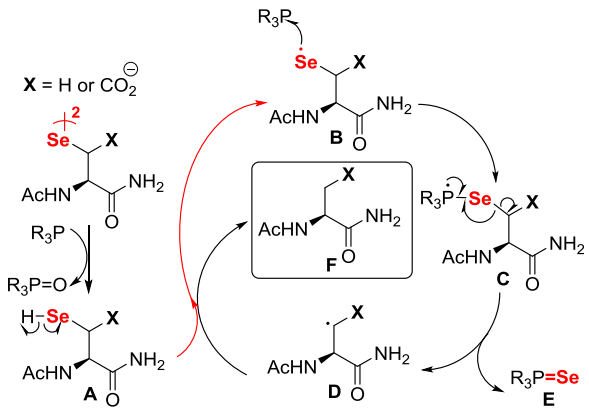
and to completion (in all cases) within 60 s, whereas deselenization at Sec requires 4–6 hr (see Figures S13–S40 for data). Interestingly, the deselenization was also complete within a minute at a wide pH range (1.8–7.0; see Figures S54–S58). The resulting products from the one-pot additive-free diselenide-selenoester 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.

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 (which normally facilitates deselenization) through the addition of ascorbic acid.[31] We were therefore interested in assessing whether (β-Se)-Asp could be used 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 Reddy et al.[31] and Figures S43 and S44), this observation reflects the increased lability of the C–Se bond in the (β-Se)-Asp moiety (see above). We therefore sought to optimize additive conditions by using an alternative radical trap, namely 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–S47). Although 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 performed strictly under an additive-free regime, we moved to further explore the exceptional rate enhancement observed for deselenization at these amino acids over 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** in 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 H-atom abstraction could be possible from selenol **A** (produced by TCEP reduction of the starting diselenide), which would regenerate **B** and propagate a radical chain as depicted in Scheme 4.

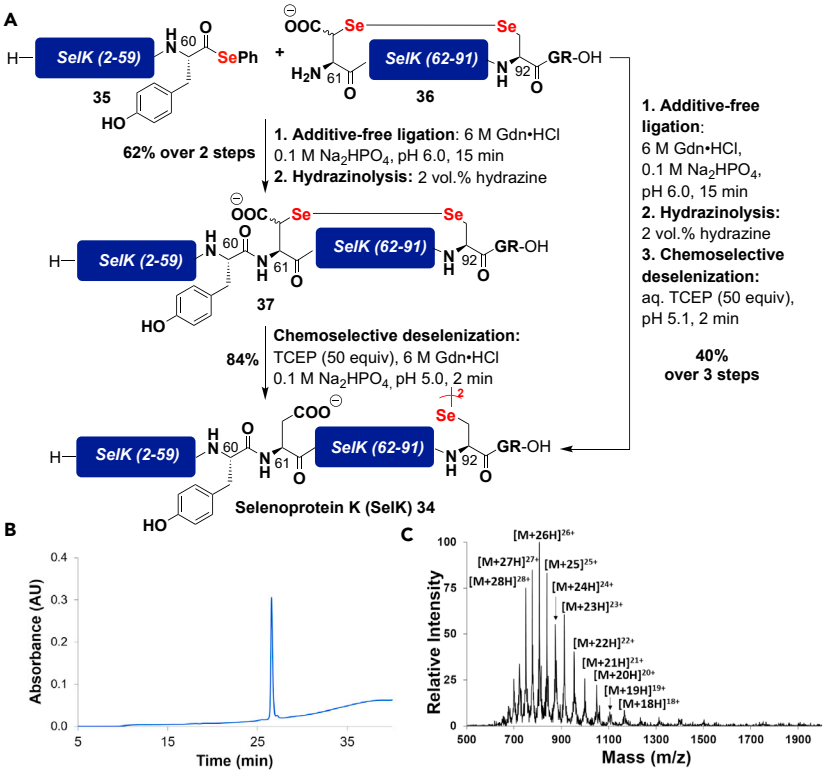


Scheme 4. Putative Pathways for the Deselenization of Selenoamino Acids
For TCEP, R = $CH_2CH_2CO_2^-$.

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] with the species depicted in Scheme 4 as models (Table S1). Gas-phase energies were obtained at the DSD-PBEP86/aug′-cc-pVTZ level,[35] and the effect of solvation was 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 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 of which indicates that this modification essentially removes the barrier (Table S1 and Figure S115). This can be attributed 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^-) than 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 homolog (Table S1 and Figure S115), which agrees with our experimental observation, i.e., that deselenization at both (β-Se)-Asp and (γ-Se)-Glu is 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. Toward 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 for assembling the protein via ligation because of its proximity to



Scheme 5. Synthesis of Selenoprotein K

(A) Synthesis of homodimeric SelK via additive-free diselenide-selenoester ligation at (β-Se)-Asp and subsequent chemoselective deselenization in the presence of native and unprotected Sec92.

(B) HPLC trace of purified synthetic SelK after the one-pot ligation-deselenization protocol (26.6 min at room temperature, λ = 220 nm).

(C) Electrospray ionization mass spectrum of synthetic SelK homodimer **34** (20,954 Da).

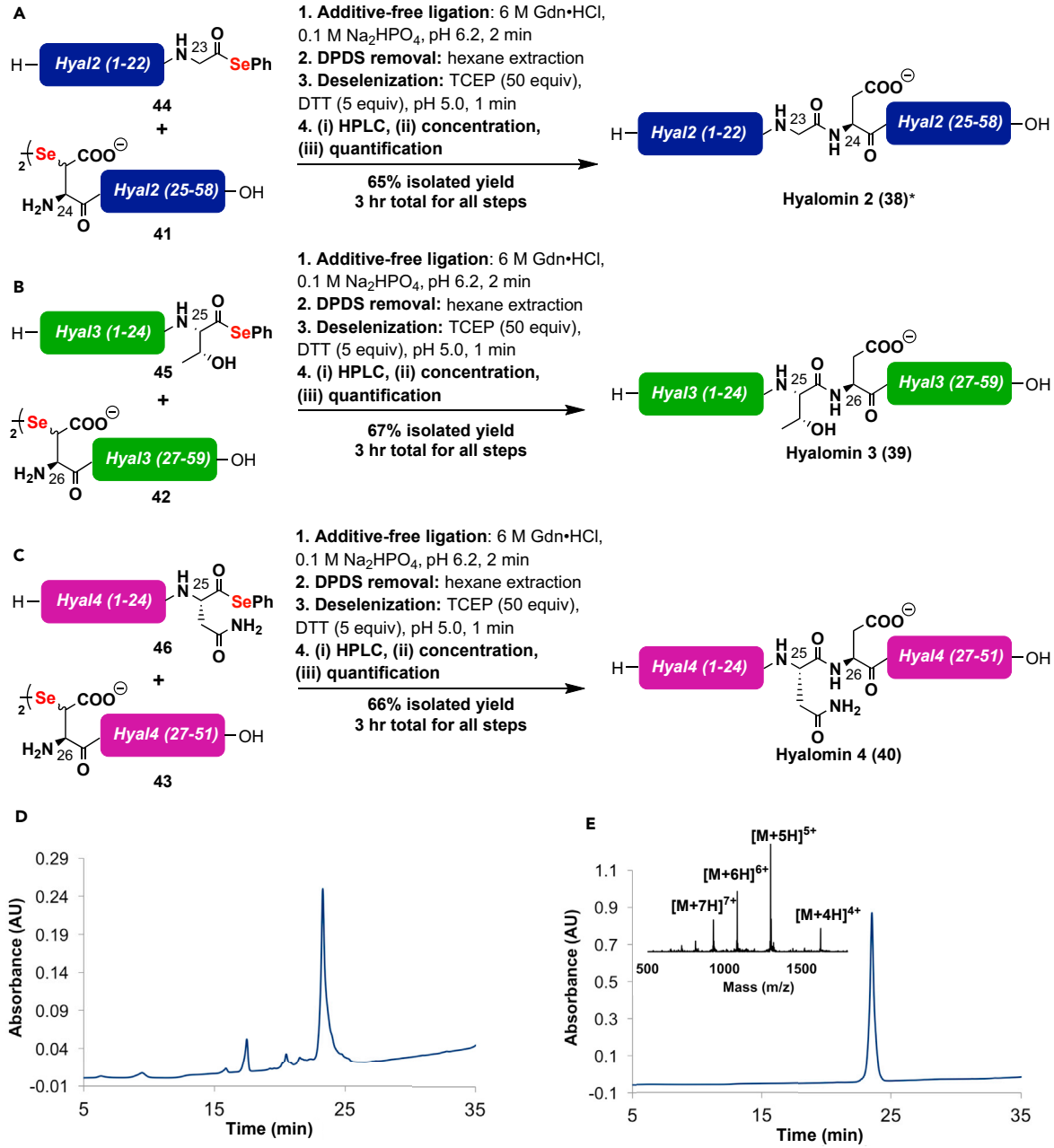
the C terminus. Unlike most selenoproteins that possess selenosulfide linkages, SelK exists as a homodimer linked via an intermolecular diselenide at Sec92.[36] Although the exact biological role of SelK has not been established, Sec92 and the high redox potential of the intermolecular diselenide bond are 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 by Fmoc-SPPS methods (Scheme 5, Supplemental 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. We performed ligation under the additive-free conditions by simply dissolving the two fragments in aqueous ligation buffer (adjusted to pH 6.0) by using a 2-fold excess of selenoester **35**. Pleasingly, the reaction proceeded to completion in 15 min (as judged by UPLC-MS analysis) to afford a mixture of intramolecular diselenide **37** and the selenoester-linked ligation product. After 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 after 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 by using a one-pot protocol (Scheme 5A). Toward 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). After reverse-phase HPLC purification, homodimeric SelK **34** was isolated in 40% yield and in excellent purity over the three synthetic steps (Schemes 5B and 5C and Figures S70 and S71).

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 hr to reach completion. 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) by 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 behavior 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 with 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).

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 time frame. Toward this end, diselenide dimer fragments **41–43** (1 equiv on the basis of the monomeric peptide) were reacted with peptide selenoesters **44–46** (1.5 equiv for **44**, 1.6 equiv for **46**, and 2 equiv for **45**) in ligation buffer (pH adjusted to 6.2) under additive-free conditions (Scheme 6). Gratifyingly, the reactions proceeded in just 2 min 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 the addition of TCEP (50 equiv) and DTT (5 equiv) and, like the model systems, proceeded smoothly and to completion within 1 min (Figures S84–S89 and S94). 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 reverse-phase HPLC over a 30 min gradient, and fractions containing the desired protein were analyzed with 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 hr at 50°C and 3 mbar. The pure proteins were re-dissolved in distilled water and the concentration confirmed by NanoDrop UV-visible spectrophotometry to determine concentrations of the protein solutions (for direct use in thrombin inhibitory assays).



Scheme 6. Rapid Assembly of Hyalomin-2, Hyalomin-3, and Hyalomin-4 via One-Pot Ligation-Deselenization Chemistry

(A–C) 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 hr time period. *Hyalomin 2 (**38**) was produced with Met18 oxidized. The sulfoxide could be reduced in a subsequent step to afford the native protein in 94% yield (see Supplemental Information for details).

(D) Crude HPLC of one-pot ligation deselenization to afford hyalomin 2 (**38**) (23.5 min at room temperature, λ = 280 nm).

(E) HPLC of purified hyalomin 2 (**38**) (23.5 min at room temperature, λ = 280 nm). Inset: the electrospray ionization mass spectrum of hyalomin 2 (**38**).

After the one-pot diselenide-selenoester ligation deselenization and purification, the hyalomins were isolated in excellent yields (65%–67%). After 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 hr 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 (half maximal inhibitory concentration = 20 μM), possibly because the C-terminal tail of this protein is significantly shorter than those of **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.

DISCUSSION

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. After 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-inhibiting proteins from the hyalomin family, which could all be assembled, purified, and isolated within 3 hr 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]$_2$ (2.0 mg, 1.4 μmol, 2.8 μmol of H-(β-Se)DSPGYS-NH_2 monomer) or diselenide dimer **12** [H-(γ-Se)ESPGYS-NH_2]$_2$ (1.0 mg, 0.7 μmol, 1.4 μmol of H-(γ-Se)ESPGYS-NH_2 monomer) and a selenoester Ac-LYRANX-SePh (**13**–**19**) (3.6–5.6 μmol for reaction with **10** or 1.8–2.8 μmol for reaction with **12**) were separately dissolved in ligation buffer (6 M Gdn•HCl and 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 was left at room temperature with intermittent agitation. Analytical HPLC-mass spectrometry (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) for the removal of 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 was 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 was left for 5 min (after 1 min of HPLC-MS analysis had 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 Supplemental Experimental Procedures, 116 figures, and 2 tables and can be found with this article online at http://dx.doi.org/10.1016/j.chempr.2017.04.003.

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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