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# Synthesising protease-stable isopeptides by proteases: an efficient biocatalytic approach on the basis of a new type of substrate mimetics†

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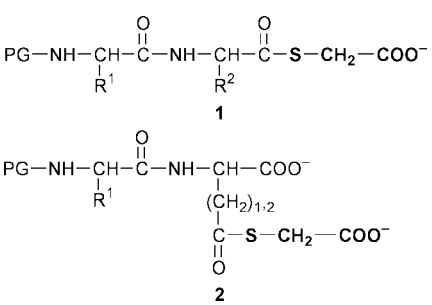
**A biocatalytic route to the 'post-synthesis' formation of Asp/Glu-derived isopeptides is illustrated on the basis of the *Staphylococcus aureus* V8 protease used as the biocatalyst, a new type of substrate mimetics as the donor peptides, and several acceptor peptides varying in length and sequence.**

The trifunctional structure of Asp and Glu allows both amino acids to be incorporated in the backbone of polypeptides by the side-chain and the $C^{\alpha}$-carboxyl moiety as well. While the latter results in the formation of normal linear peptides, the involvement of the side-chain carboxyl group in the peptide backbone leads to the corresponding isopeptides. Isopeptides are ubiquitously found in nature and are considered to be one of the most common forms of non-enzymatic degradation of linear polypeptides under physiological conditions.[1] Rearrangement of normal peptides into isopeptides is catalysed by bases as well as acids, and starts with the formation of cyclic succinimide intermediates following dehydration. Re-opening of the ring finally leads to a mixture of normal peptides and isopeptides in a typical ratio that ranges from 40 : 60 to 15 : 85 along with small amounts of D-isopeptides.[2] Since isopeptide formation involves the addition of extra carbon atoms to the polypeptide backbone, its presence in a functional protein domain has profound effects on biological activity and its susceptibility to proteolytic degradation.[3] Recent findings have demonstrated that widespread accumulation of isopeptide linkages in cellular proteins greatly increases the immunogenicity and disrupts a wide range of important biochemical pathways by competitively displacing normal proteins in protein–protein or protein–ligand interactions.[4] Because of this function, isopeptides were found to be highly useful to study complex biological phenomena and for the development of therapeutic agents resulting in a great interest in their synthesis.

Although isopeptide bond formation is often an undesired side reaction during the chemical synthesis of normal linear peptides due to the need for strong bases and acids for deprotection steps and peptide release,[5] the directed synthesis of isopeptides is difficult and strictly limited by competitive cyclisation reactions. Like bases and acids, the use of coupling reagents to yield side-chain activation of Glu- and Asp-containing peptides further reinforces the formation of unwanted cyclic products and finally leads to a crude synthesis product with substantial amounts of undesired material. A few enzymes, *i.e.* protein carboxyl methyltransferases,[6] isopeptidases,[7] and transglutaminases,[8] are known to be active on the side-chain of Glu and Asp and, therefore, may be interesting biocatalysts for the synthesis of isopeptide bonds. However, due to the highly restricted substrate and reaction specificity, none of these enzymes is capable of catalysing the synthesis of isoAsp- or isoGlu-Xaa bonds. Therefore, the development of alternative catalytic and mild approaches to improve the current synthesis methods of isopeptides is a challenging task.

This account reports on the use of a normal peptidase, *i.e.* the Glu-specific endopeptidase from *Staphylococcus aureus* strain V8 (V8 protease), that acts as an efficient biocatalyst for the formation of isopeptides. The key feature of this approach is the use of a novel type of substrate mimetics that directs the synthesis activity of the protease to the side-chain carboxyl moiety of Asp and Glu (Scheme 1). Similar to classical substrate mimetics, the novel type donors bear a site-specific ester leaving group that mediates the acceptance of non-specific acyl moieties by the protease.[9] To direct the activity of the enzyme to the side-chain, the specific ester group, however, is linked to the ω-carboxyl moiety of Asp and Glu instead of being at the C-terminus of the peptide donor. This different architecture was shown to lead to a shift in the synthetic activity of the protease from the $C^{\alpha}$-carboxyl group towards the side-chain moiety of the two amino acids finally resulting in the synthesis of isopeptides.

The general function of this biocatalytic approach was proved by the use of several Asp- and Glu-containing peptides that were esterified by carboxymethylthiol at their side-chain carboxylates. The carboxymethyl thioester functionality (SCm, *cf.* Scheme 1) itself was selected due to its function as a mimic which was already shown to mediate the acceptance of non-specific (non-Asp- and Glu-containing) linear donor peptides by V8 protease and other highly Glu-specific peptidases as well.[10] The influence of the length of the donor peptide on the synthesis reaction was investigated by using donor esters derived from single amino acids up to pentapeptides. Furthermore, the effect of the position of the Asp and Glu moieties within the donor peptide on the acceptance by the enzyme was studied by using peptides with either N-/C-terminal or endogenous Glu/Asp-residues. The resulting 14 peptide esters have served as donor components in V8 protease-catalysed isopeptide syntheses using amino acid amides, dipeptide amides, and




**Scheme 1** General structures of classical linear (**1**) and new type (**2**) substrate mimetics. The site-specific carboxymethyl thioester group is emphasized by bold letters. PG, protecting group; $R^1$, $R^2$, individual side chains.

† Electronic supplementary information (ESI) available: complete details for experimental procedures. See http://www.rsc.org/suppdata/cc/b1/b105752a/

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**Table 1** Yields (%) of the V8 protease-catalysed synthesis of isopeptides. [donor]: 2 mM, [acceptor]: 15 mM, [V8 protease]: 3.61–10.33 μM

| | Acceptor peptide | | | | |
|---|---|---|---|---|---|
| Donor peptide | H-Met-$NH_2$ | H-Gly-Leu-$NH_2$ | H-Leu-Gly-$NH_2$ | Ile-Ala-Ala-Ala-Gly | Leu-Ala-Ala-Ala-Gly |
| Z-Glu/*Asp*(SCm)-OH | 41.2/*41.5* | 40.3/*41.6* | 47.7/*48.5* | 49.8/*49.3* | 50.5/*52.2* |
| Z-Ala-Glu/*Asp*(SCm)-$NH_2$ | 41.3/*59.5* | 39.8/*56.9* | 47.0/*60.3* | 47.2/*67.9* | 50.3/*65.9* |
| Z-Ala-Ala-Glu/*Asp*(SCm)-$NH_2$ | 44.9/*53.9* | 45.1/*56.6* | 50.4/*61.9* | 49.4/*62.3* | 51.0/*61.8* |
| Z-Glu/*Asp*(SCm)-Ala-$NH_2$ | 39.0/*51.7* | 39.6/*54.2* | 46.5/*59.7* | 48.6/*66.8* | 49.3/*64.3* |
| Z-Glu/*Asp*(SCm)-Ala-Ala-$NH_2$ | 44.7/*58.7* | 45.4/*55.8* | 52.6/*61.7* | 54.6/*67.5* | 54.9/*64.8* |
| Z-Ala-Glu/*Asp*(SCm)-Ala-$NH_2$ | 42.5/*60.9* | 43.9/*62.8* | 50.2/*65.7* | 50.1/*67.4* | 51.6/*68.0* |
| Z-Ala-Ala-Glu/*Asp*(SCm)-Ala-Ala-$NH_2$ | 40.8/*59.1* | 41.5/*61.9* | 51.9/*64.0* | 52.4/*66.4* | 52.8/*65.0* |

pentapeptides as the amino components. The reactions themselves were performed under identical conditions at pH and temperature optimal to the enzyme.[11] As a control for spontaneous hydrolysis and aminolysis of the peptide esters that may interfere with the enzymatic syntheses, parallel reactions without enzyme were analysed. On the basis of these control experiments, non-enzymatic aminolysis could be ruled out and the extent of spontaneous hydrolysis was found to be less than 5%. The results observed for the enzyme-catalysed syntheses are summarised in Table 1. Generally, the data document that all donor peptides show productive binding at the active site of the biocatalyst resulting in isopeptide bond formation. In contrast, model reactions using donor components lacking the specific ester moiety, *i.e.* Z-Glu(SMe)-OH and Z-Asp(SMe)-OH (SMe, methylthiol), gave no reaction. This finding indicates that the negative charge of the SCm-leaving group is essential to mediate the acceptance by V8 protease. Accordingly, the lack of this charge causes a complete loss of specificity that inevitably leads to a loss of synthesis activity of the enzyme. On analysis of the efficiency of syntheses, yields of isopeptide products within a range of about 40–70% were obtained that roughly correspond with those of comparable reactions using normal linear substrate mimetics.[10] Interestingly, apart from the formation of the respective hydrolysed donor peptides no further side products could be detected. Addressing the moderate variations in the yields, the Asp- and Glu-residue itself appears to affect the efficiency of synthesis to the highest extent. While the use of Z-Glu(SCm)-OH and Z-Asp(SCm)-OH led to practically the same yields, the chain elongation of the Asp ester either in the C- or N-terminal direction resulted in an increase in the product yields of around approximately 10–20%. This increase makes the Asp-containing peptides more efficient donor components than those derived from glutamic acid. Interestingly, the individual position of both the Asp and Glu residue within the donor peptide does not affect the course of isopeptide synthesis significantly. In the same way, there is only a minor influence of the length of the donor peptide on the reaction. Surprisingly, this also holds for the acceptor peptide as it is reflected by the similar product yields which are less affected by the sequence and the chain length of the respective peptide. This atypical behaviour is in contrast to reactions using classical linear substrate mimetics as the donor peptides that generally displayed a more pronounced influence of the acceptor peptide on the course of synthesis.[10] Accordingly, in particular, N-terminal Gly and Met residues usually led to a decrease in product yield; an effect that can not be found for the synthesis of isopeptides. The synthesis of glutathione (γ-glutamylcyteinylglycine) starting from Z-Glu(SCm)-OH and Cys-Gly, which proceeds with a yield of about 62%, gave a further hint to the small influence of the acceptor peptide on the efficiency of catalysis. From a synthetic point of view, this broad tolerance towards the sequence and chain length of both the acceptor and donor peptide should make the approach presented a rather general one for the synthesis of a wide variety of isoaspartyl- and isoglutamyl-containing peptides. Furthermore, due to the mild reaction conditions, the weak carboxyl activation, and abandoning the use of additional bases and acids, the risk of undesired cyclisation reactions is reduced to a minimum. The synthetic utility of the method is even not narrowed by proteolytic side reactions. Model syntheses with elongated reaction times up to several days do not gave any hints to an undesired cleavage activity of V8 protease towards the formed isoGlu- and isoAsp-bonds indicating an irreversible course of the enzymatic ligation reaction (data not shown). Only the presence of further Glu and Asp residues additional to those that are to be modified may lead to undesired peptide cleavages. In such cases, freezing of the reaction mixture, which was shown to repress the proteolytic activity of enzymes closely related to V8 protease while retaining their synthesis activity,[12] may represent an efficient resource. From a biocatalytic point of view, the approach presented broadens the scope of proteases in organic synthesis and opens up a new field of synthetic applications of these enzymes for the synthesis of isopeptides. Finally, because of the universal applicability of substrate mimetics it can be further expected that other proteases may also be useful biocatalysts for isopeptide synthesis. Studies in this direction are presently under investigation.

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