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BIOMIMETIC PROTEASE PRODUCTION SYSTEM, DEVELOPED

DELIVERABLE NUMBER D4.4

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Document information sheet

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Summary

Biomimetic protease production system, developed

1. Scope of Deliverable

This deliverable consists in a green chemical-system by which to metamorphose, through the use of catalytic suicide inhibitors, esterases capable to be produced at high yields, into versatile biomimetic proteolytic systems. This deliverable is accompanied by a report detailing the protocol of design and use, which will be made available in the internal FuturEnzyme repository.

The work for this deliverable corresponds to Task 4.2_Smart design systems to obtain enzymes with inherent problems of expression (M2-M30), led by FHNW and with the participation of CSIC and Eucodis. The due date has been asked to be postponed (*see* section 5), although in this document, delivered on the first due date, the work accomplished so far is detailed.

2. Introduction

Proteases are pivotal enzymes for the hydrolysis of peptide bonds in materials where proteins are abundant components and are widely used also in organic synthesis. This is why they constitute 60–65% of the global industrial market, growing at an annual growth rate of 5.6%. Through evolution, proteases have adapted to the wide range of conditions found in complex organisms (variations in pH, reductive environment and so on) and use different catalytic mechanisms for substrate hydrolysis; their mechanism of action classifies them as either serine, cysteine or threonine proteases (amino-terminal nucleophile hydrolases), or as aspartic, metallo and glutamic proteases (with glutamic proteases being the only subtype not found in mammals so far). Proteases specifically cleave protein substrates either from the N or C termini (aminopeptidases and carboxypeptidases, respectively) and/or in the middle of the molecule (endopeptidases). Proteases can be easily screen, by functional screens or in silico predictions, in microorganisms or microbial communities by applying genomics and metagenomics approaches. Of course, it should be stressed that novelty itself does not guarantee better enzymatic performance and better opportunities of commercialization. Whatever the methods applied for discovery, unlocking the biochemical or biotechnological potential of sequences encoding proteases, however, requires laborious wet lab work, including extensive cloning of genes of interest, followed by the expression and characterization of enzymes. This is not a trivial exercise given that not all genes in a genome or a metagenome can be successfully cloned and expressed. This is especially important in the cases of proteases whose suffers major problems of expression, compared to other types of enzymes that are as easy to be screened as proteases but better to be produced at high levels. As example, proteases are abundant in prokaryotic genomes, about 10 per genome, but their recovery encounters expression problems, as only 1% can be produced at high levels; this value differs from that of similarly abundant esterases (1-15 per genome), 50% of which can be expressed at good levels.

As a continuation of the above, proteases are used in a broad range of applications, including bio-refinery targeting a broad range of biomasses. They have also a pivotal role in detergents, being essential components in the detergent formulations, a capacity that is being used in FuturEnzyme, where protease constitute the second type of priority enzymes after lipases (see Deliverable 2.1). In the project, and in particular in this deliverable, we aim to solve the protease expression problem we encountered through genome and metagenome screens by transforming esterases that are easy to search for and produce into proteases by means of a chemo-catalytic system that can be used to endow esterases with proteolytic activity. At this point it has to be considered that in the case of liquid detergents, the cost of enzymes should be as low as possible given that the cost of the final product is low. Therefore, this deliverable will contribute to the efficient design and production of biomimetics with protease activity.

3. Our model: natural cysteine proteases

Papain is a cysteine protease that has been thoroughly studied; it is used in many applications including cosmetic, health, food, and pharmaceutical industries. We decided to use papain as our model enzyme and inspiration to design a chemo-catalytic system that can be used to endow esterases with proteolytic activity; **Figure 1**.

Besides catalytic activity, the new molecular entities produced are expected to possess and esterase inhibitor activity to precisely home in the active site of the enzyme. Accordingly, an inhibitor molecule will be synthesized and later be incorporated to an enzyme. For the synthesis, cysteine and histidine amino acid will be inserted to an indole backbone.

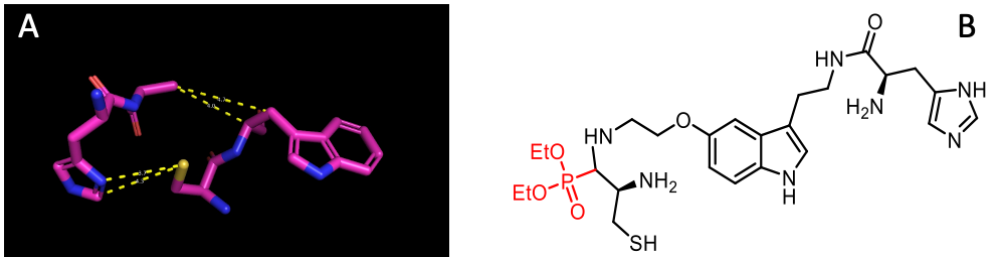
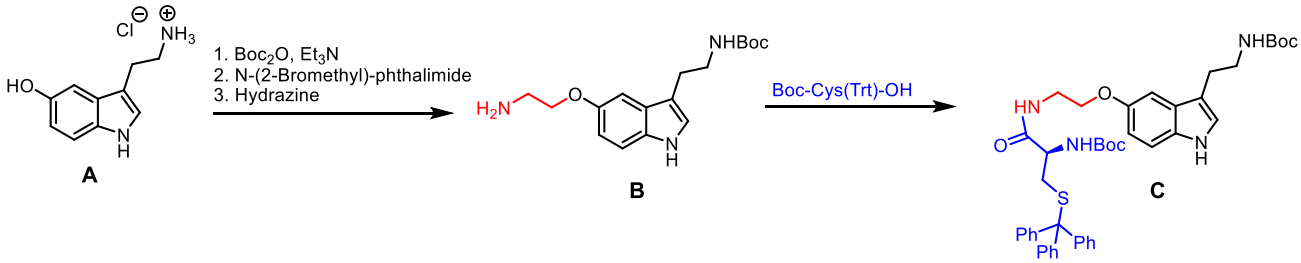


Figure 1: Active site of papain (A) and target biomimetic catalytic inhibitor molecule (B)

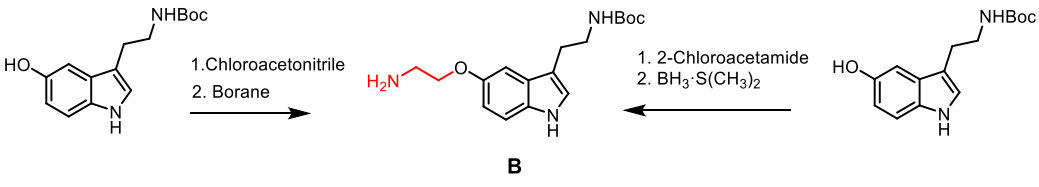
4. Synthetic strategy

Serotonin **A** was used as a starting material to minimize the total number of steps. Initially, serotonin has been protected with a *tert*-Butyloxycarbonyl (Boc) protecting group on the primary amine, to allow for a Gabriel reaction, namely the nucleophilic substitution with a phthalimide derivative followed by a reduction using hydrazine to yield compound **B**, **Scheme 1**. Except for the first step, all reaction products are new and optimization work was required. The next step planned was the addition of cysteine protected via an established peptide coupling method to obtain **C**.



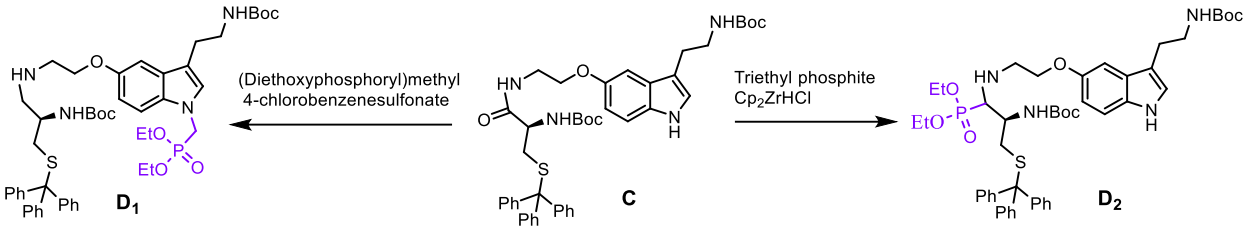
Scheme 1: First steps from serotonin starting material

Unexpectedly, the Gabriel reaction followed by the release of the primary amine in presence of hydrazine didn't yield the expected product **B**; an alternative synthesis method was proposed. By using the same starting material as in the first step, an SN_2 type reaction can be performed to produce a terminal nitrile which will be later reduced in presence of a Borane in THF (**Scheme 2**). Another possibility is the use an acetamide which will be reduced with borane dimethyl sulfide complex to afford the product **B**.



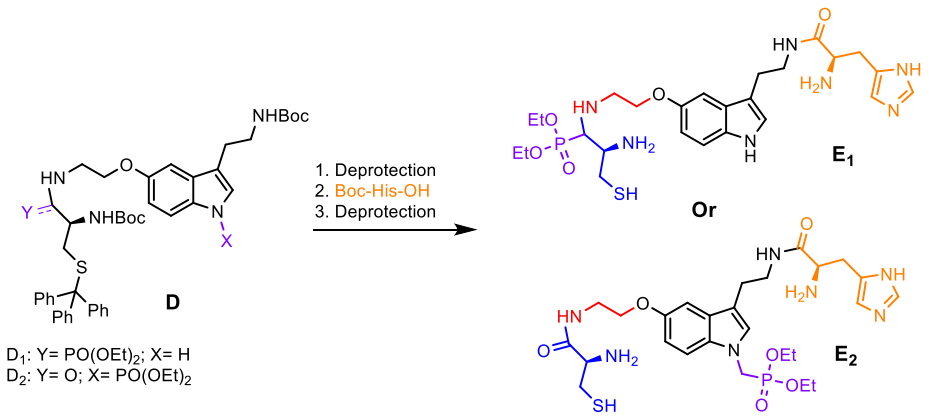
Scheme 2: Alternative synthesis from Boc protected serotonin

The insertion of the inhibitor to the enzyme requires a phosphonate ester group within the molecule. It was decided to carry out this reaction after the amide with protected cysteine since none of the functional groups are expected to interfere with the reaction (**Scheme 3**). Two phosphonate addition methods are planned. In the first approach, in the presence of a zirconium complex, a phosphonate can be used to replace the carbonyl of the amide bond of the cysteine residue. The second method is based on the cleavage of the phosphonate diesters on the indole derivative.



Scheme 3: Insertion of phosphonate group

The next step shown in **Scheme 4** is the deprotection of the two Boc group in **D** to yield a molecule bearing a secondary and a primary amine, which will later be converted to an amide via peptide coupling. In the final last step, a full deprotection of the trityl protector group from cysteine and Boc group of the histidine are planned. Depending on the phosphonate used (**E1** or **E2**) will provide two inhibitors and will enrich a library of catalytic inhibitors.



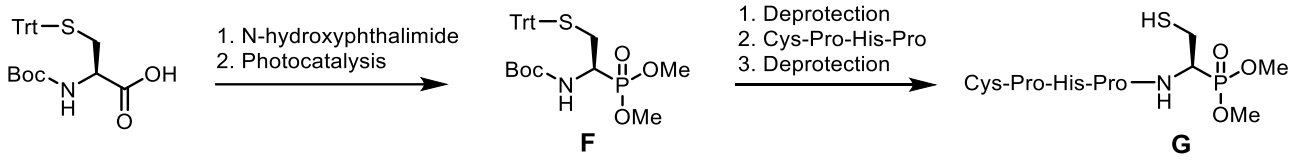
Scheme 4: Last steps synthesis to achieve two catalytic inhibitors E1 and E2

5. Biochemically attaching a short catalytic peptide to the active site of an esterase

Besides the main synthetic approach described above, we also plan to produce short catalytic peptides that can be docked into the esterase active site via covalent inhibition. Several peptides designs were submitted to geometric and computational quantum mechanical modelling method (DFT, density functional theory) in order to evaluate their suitability to mimic the catalytic triad of proteases. It is known that in the active site of papain, the distance between His and Cys is crucial to increase the nucleophilicity of the thiol function and to allow for the formation of a thioester intermediate. Different peptide sequences were tested and one, namely Cys-Pro-His-Pro-Cys, showed satisfactory results. This peptide will be produced using solid-phase synthesis.

In addition to the peptide synthesis, we need to introduce at *C*-terminus end of the peptide, a phosphonate group that will allow the covalent docking in the active site of the esterase. To that end, we will apply a method that has been recently published by Aggarwal *et al.*, which allows transforming alpha amino acids

into alpha amino phosphonate by using visible Light photocatalysis.[1] This method will be applied to our peptide sequence, **Scheme 5**.



Scheme 5: Planned peptide phosphorylation

6. Comments on due date

There have been difficulties regarding human resources that have forced us to not fully complete this deliverable on time. In an online meeting that the Coordinator (Manuel Ferrer) held with the Project Officer (Colombe Warin), on 19th September 2022, this point was discussed. In the present document, we have summarized the activities done in the frame of this deliverable until know and below we reflect the causes of the delay, explain impacts if any, and propose a new argued due date.

At the beginning of FuturEnzyme (June 2021), FHNW hired a new scientist to work on the project. Because of difficulties (also related to COVID-19 travel restrictions), this took longer than planned. Giada Sabatino was hired to do the work, and started at 1st October 2021. She got sick at the end of December and did not work the whole month of January. She then decided to resign from her position. The work carried out by Ms. Sabatino during the short period she worked on the project is reflected in the sections above. After a new selection procedure, FHNW hired a synthetic chemist, Dr. Guillaume Magnin, to carry out the experimental work, who began FuturEnzyme's activities in July 2022 and continued with the work, obtaining results detailed above.

This deliverable corresponds to Task 4.2, which is planned from month 2 to 30 (July 2021 - November 2023); despite of this, there is only one deliverable (the present one, D4.4) at month 16 (September 2022). This means that the work carried out from months 17 to 30 might not be reported if we fully complete the present deliverable in month 16. For this same reason, no impact is expected on the overall implementation of the project. This no or little impact is also defined by the fact that during the first year of the project, lipases have been prioritized as the main target by our industrial partner, Henkel, and proteases as a secondary target (see deliverable D2.1); we therefore consider that this extra time would not represent any inconvenience. In addition, some of the lipases that are currently being identified and tested as being of high interest could be the basis in the coming months for the design of biomimetics with protease activity in the frame of the activities covered in D4.4. Taking this into account, plus the personnel difficulties faced by FHNW, the new due date for this deliverable 4.4 is proposed to be postponed until month 30 (November 2023), at the end of the corresponding Task 4.2.

[1] D. Reich, A. Noble, V. K. Aggarwal, *Angew. Chem. Int. Ed.* **2022**, *61*, e202207063