**Applications of enzymes and membrane processes in peptide production**

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This PhD project aims to develop an immobilized enzyme bioreactor coupled to an electrodialysis with ultrafiltration membranes system for the production and fractionation of peptides from the hydrolysis of whey proteins.

**Applicazioni di enzimi e processi a membrana nella produzione di peptidi**

Questo progetto di dottorato mira allo sviluppo di un bioreattore ad enzima immobilizzato accoppiato ad un sistema di elettrodialisi con membrane di ultrafiltrazione per la produzione e il frazionamento di peptidi mediante idrolisi di sieroproteine.

**1. State-of-the-Art**

Proteins, as a result of the cleavage of peptide bonds, are broken down into peptides of different sizes and free amino acids. This degradation, termed hydrolysis, can be carried out by enzymes (proteolysis), acids or alkali. Acid and alkaline hydrolysis tends to be a difficult process to control and yields products with reduced nutritional qualities. Enzymatic hydrolysis is developed under mild conditions of pH (6-8) and temperature (40-60° C), avoiding the extremes usually required for chemical and physical treatments and minimizing side reactions (Clemente, 2000). Proteolysis is becoming a method of choice in the food and pharmaceutical industries. Peptides derived from proteolysis can be used to develop nutraceuticals or functional foods with improved biological functions such as antioxidant, antihypertensive, antimicrobial activities, among others (García *et al.*, 2022). In addition to the classical hydrolysis using free enzymes, in recent years, research has been oriented towards the use of enzymes immobilized on various supports which has some advantages such as increased enzyme stability, enzyme re-cycle, use of high enzyme to substrate ratios. However, for the latter, it is crucial to select the appropriate immobilization supports having a high superficial density of reactive groups, as well as suitable immobilization conditions, such as reaction time, pH, temperature, buffers, and inhibitors or protein protectors, to enhance the enzyme–support reaction (Bortone *et al.*, 2012).

A protein hydrolysate contains many peptides of which only some have a biological activity. Therefore, there is a need to identify and characterize the latter, which begins with their fractionation. The main drawback of peptide fractionation is that most of the peptides share very similar physicochemical characteristics, therefore, only a separation technology able to distinguish between subtle differences in charge, size, solubility or hydropathicity results of utility (Fernández *et al.*, 2013). Over the years, various peptide fractionation processes have been studied such as chromatographic processes (Wafaa *et al.*, 2022) and membrane technologies. The latter is a low-cost, environmentally friendly technology, which works under mild operating conditions and leave the substrate nutritional properties almost intact. Among membrane processes, nanofiltration is considered as especially appropriate for peptide separation, due to the molecular weight cut-off used and the importance of charge effects. The combination of nanofiltration and ultrafiltration has also been used (Arrutia *et al.*, 2016). On the other hand, electrodialysis with ultrafiltration membranes which is an electromembrane process is gaining increasing interest for the recovery of charged molecules, especially bioactive peptides as part of sustainable strategies (Geoffroy *et al.*, 2022).

So far, the production of bioactive peptides has mostly been operated in batch mode with free enzyme. This presents some disadvantages such as the higher energy consumption due to the long processing time, the product accumulation that acts as an inhibitor of the hydrolysis (Sousa *et al.*, 2004) and the fact that the enzyme can be only used once. To overcome these problems, this PhD project will be directed to develop a more viable system based on an immobilized enzyme bioreactor to be coupled with an electrodialysis with ultrafiltration membranes system to continuously produce bioactive peptides from whey proteins with the advantages of lowering the number of unit operations, the amount of enzyme used and the energy consumption.

# **2. PhD Thesis Objectives and Milestones**

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Immobilization studies.** Screening of the scientific literature for available commercial enzymes for whey protein hydrolysis (A1.1). Choice of the enzyme/enzymes to use based on several aspects (degree of hydrolysis, potential bioactivity of peptides released) (A1.2). Development of the immobilization process/processes (A1.3). Characterization of the obtained immobilized biocatalysts (immobilization yield, immobilized activity, stability) (A1.4).

A2) **Bioreactor development.** Set up of an immobilized enzyme bioreactor (A2.1). Study of operating conditions and reactor configuration on hydrolysis degree (A 2.2).

A3) **Development and testing of electrodialysis system.** Identification of the ED with ultrafiltration membranes configuration and setting up of the system (A3.1). Separation tests with protein hydrolysate produced by the enzymatic reactor (A3.2). Development of the combined reactor with the electrodialysis system and separation tests (A3.3). Characterization of separated fractions of peptides (A3.4).

A4) **Design of experiments, development of empirical and/or mechanistic models of the system and data analysis.** Design of experiments (A4.1). Modeling of the enzyme kinetics for free and immobilized systems (A4.2). Basic modeling of the electrodialysis system (A4.3). Data analysis through univariate and multivariate techniques (A4.4).

A5) **Writing and Editing** of the PhD thesis, scientific papers, oral and/or poster communications.

***Table 1***Gantt diagram for this PhD thesis project.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Activity Months | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** | **20** | **21** | **22** | **23** | **24** | **30** |
| A1) | ***Immobilization studies*** |   |   |   |  |  |  |  |   |   |  |  |  |  |   |  |  |  |  |   |   |  |  |  |  |  |
|  | 1) Selection of appropriate enzymes |   |  |   |  |  |  |  |   |   |  |  |  |  |   |  |  |  |  |   |   |  |  |  |  |  |
|  | 2) Studies of enzymes characteristics  |   |  |   |  |  |  |  |   |   |  |  |  |  |   |  |  |  |  |   |   |  |  |  |  |  |
|  | 3) Immobilization process development |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | 4) Biocatalyst characterization  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| A2) | ***Bioreactor development*** |   |   |   |  |  |  |  |   |   |  |  |  |  |   |   |   |  |   |   |   |  |  |  |  |  |
|  | 1) Set up of immobilized enzyme bioreactor |   |   |   |  |  |  |  |   |   |  |  |  |  |   |   |  |  |  |   |   |  |  |  |  |  |
| 2) Study of hydrolysis degree  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| A3) | ***Development and testing of electrodialysis system*** |   |  |   |   |   |   |  |   |   |   |   |   |  |   |  |  |  |  |   |   |   |   |   |  |  |
|  | 1) Set up of the electrodialysis system |   |  |   |   |   |  |  |   |   |   |   |  |  |   |  |  |  |  |   |   |   |   |  |  |  |
| 2) Separation tests of protein hydrolysis |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | 3) Separation with the combined reactor with ultrafiltration system |   |  |   |  |  |   |  |   |   |  |  |   |  |   |  |  |  |  |   |   |  |  |   |  |  |
| 4) Characterization of separated fractions |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| A4) | ***Development of empirical and/or mechanistic models of the system and data analysis***  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | 1) Design of experiments |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 2) Modeling of the enzyme kinetics |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 3) Modeling of the ED system |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| 4) Data analysis |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| A5) | ***Thesis and Paper Preparation*** |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |  |   |   |   |   |   |   |   |  |

# **3. Selected References**

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