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# Understanding proteases' mechanisms for production of pea protein hydrolysates

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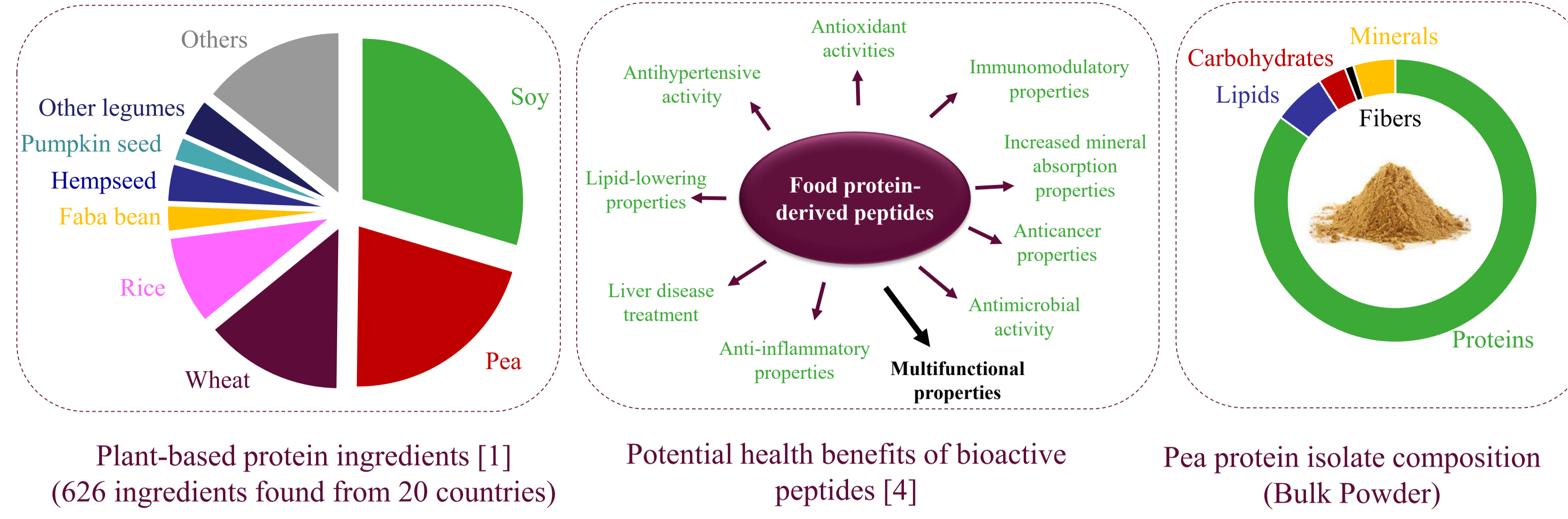
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## Introduction

Plant-based proteins are arising as an interesting alternative to their animal counterparts, due to their contribution to food system sustainability. Among plant sources, pea proteins represent more than 20% [1]. Such a success can be explained by their health benefits, as they contain interesting amounts of essential amino acids. Unfortunately, pea proteins have poor functional properties which can limit their use in food products but solubility can be highly improved by controlled enzymatic hydrolysis [2, 3]. Moreover, the peptides released by enzymatic action can also have interesting bioactive properties [4]. Many enzyme preparations are on the market for them to be used in food products. They consist of a mixture of activities (endoprotease, exoprotease) in order to achieve efficient protein hydrolysis [5]. Therefore, it can be difficult to control the use of these preparations and achieve a selective peptide production. The understanding of enzymes' mechanism will help to control selective production of peptides with interesting activities or protein functional properties modifications. The aim of this work is to investigate the activities of two commonly used food-grade proteases: Alcalase and Flavourzyme.

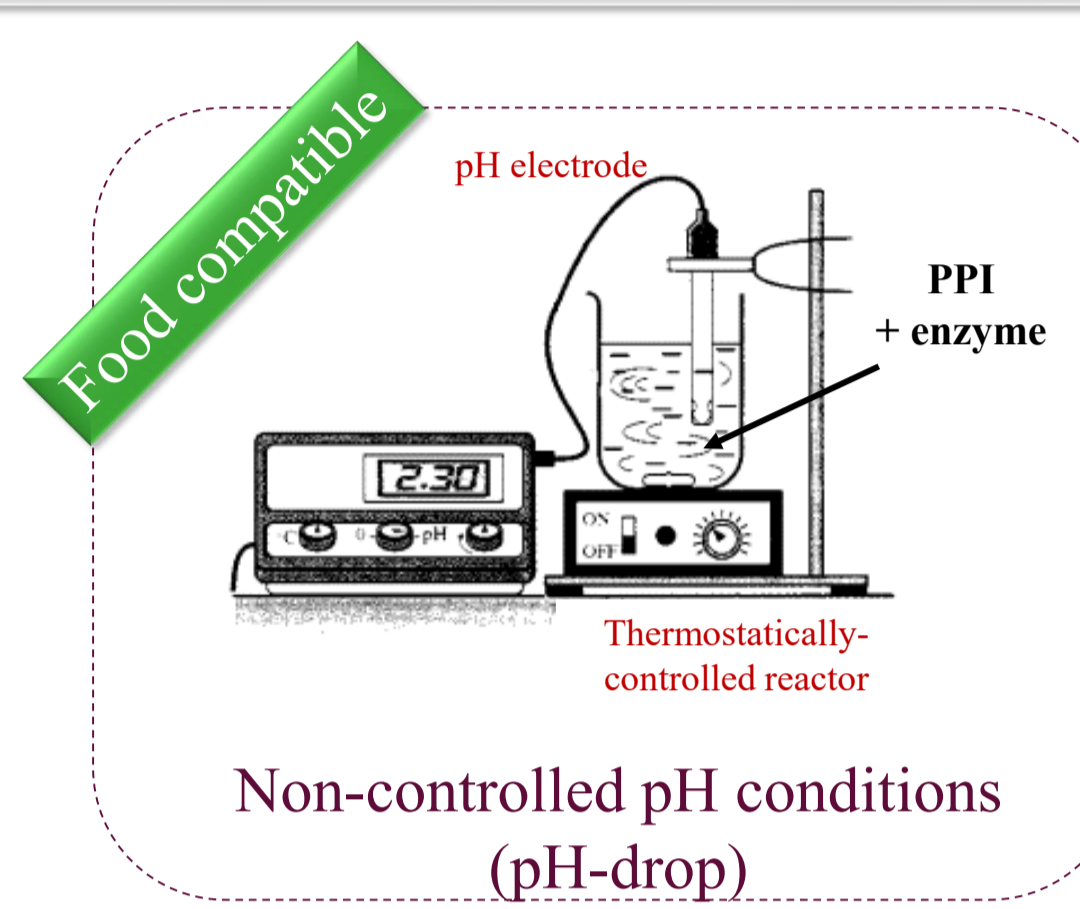


## Material and methods

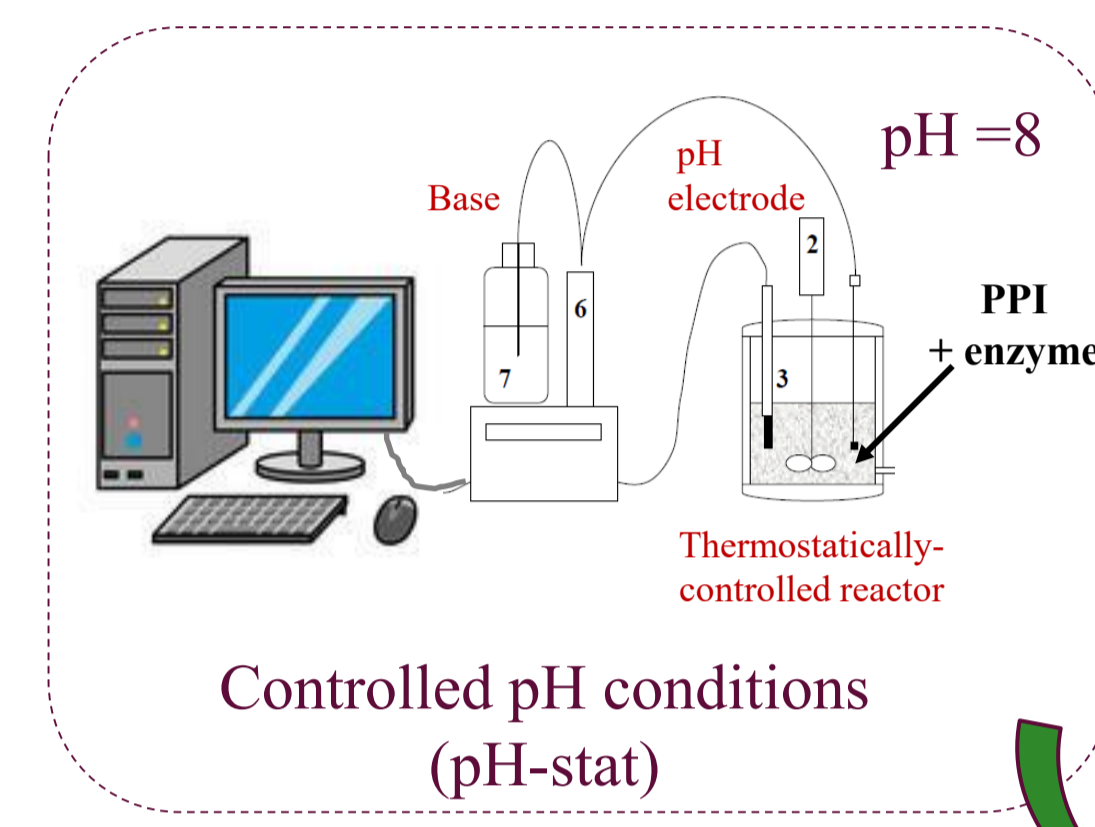
### Pea protein isolate (PPI\*) hydrolysis using Flavourzyme® or Alcalase®

	Flavourzyme (FL)	Alcalase (AL)
Commercial name	Flavourzyme® 1000L	Alcalase®
Type of protease	Mainly exoprotease (aminopeptidase, EC 3.4.11) Small proportion of endopeptidase (3.4.24)	Endoprotease (Subtilisin) (EC 3.4.21.62)
Family	Metalloprotease (neutral protease)	Serine protease (alkaline protease)
Cleavage specificity	Mainly after Leu and Pro residues	Carboxyl side of hydrophobic amino acids
Optimum pH	7.0	8.0
Optimum temperature (°C)	50-55	50-60
Enzyme inactivation	100 °C – 15 min	100 °C – 15 min

\*PPI: Super pea protein isolate from yellow pea (Bulk Powders)



Non-controlled pH conditions (pH-drop)



Controlled pH conditions (pH-stat)

Two-hours reaction, 50 ° C, Enzyme/Substrate = 238 µL.g<sup>-1</sup>

### Calculation of the degree of hydrolysis (DH)

$$DH(\%) = \frac{B * N}{\alpha * m_p * h_{total}} * 100$$

DH : degree of hydrolysis (%)  
B : Base volume (mL)  
N : Base normality (N)  
 $\alpha$  : Mean dissociation coefficient of  $\alpha$ -aminated groups  
 $m_p$  : amount of protein (g)  
 $h_{total}$  : peptidic bounds in the substrate (meqv/g protein)

### Peptides characterisation

- **Peptide's molecular weight distribution** (size exclusion chromatography)  
Yara SEC2000 column, isocratic elution (phosphate buffer pH 6.9), detection at 214 nm (peptide bond).

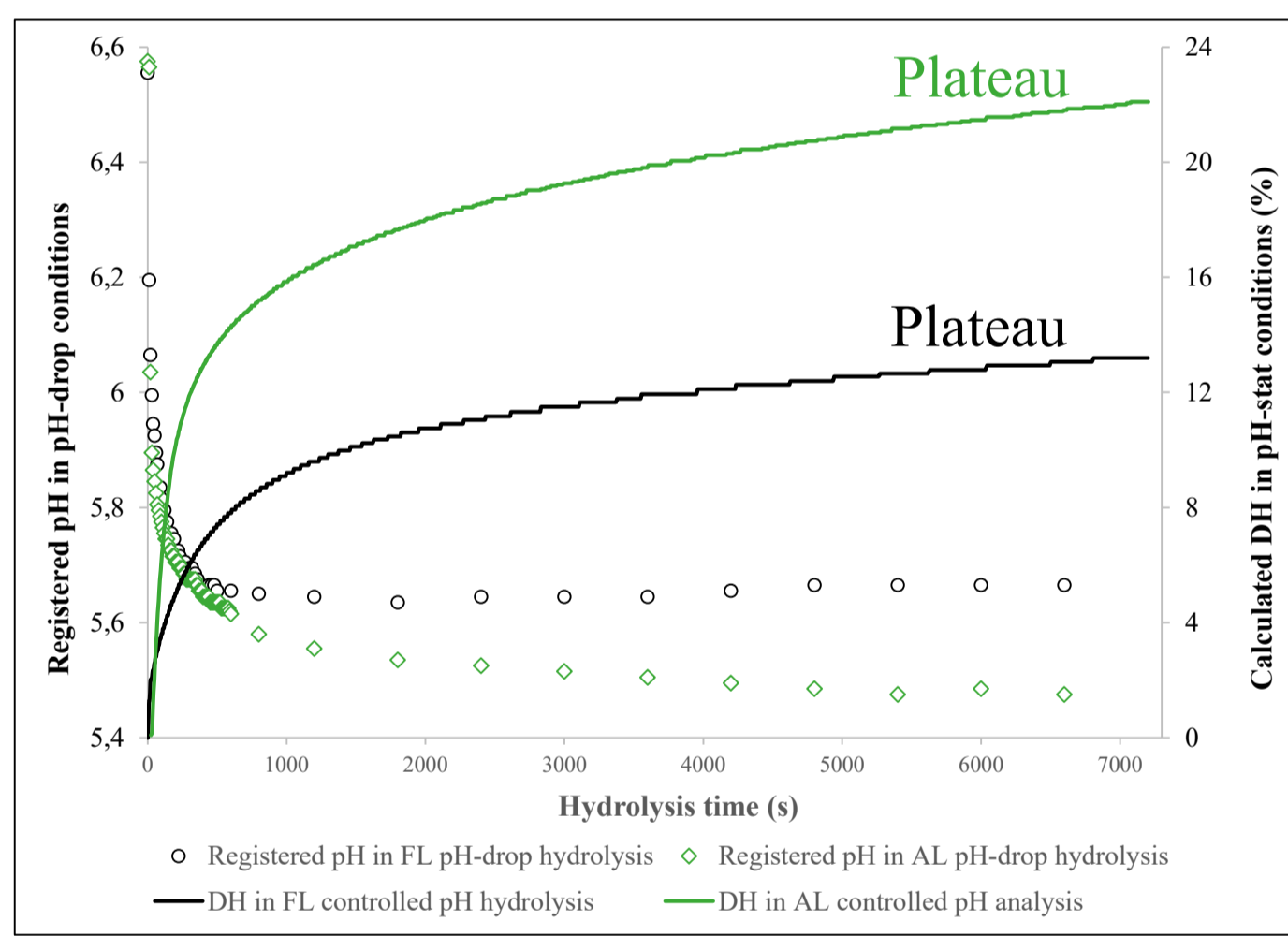
- **Antioxidant properties** : Ferric Reducing Ability of Plasma assay (FRAP)  
Reducing power was determined using potassium ferrocyanide and an absorbance measurement at 700 nm [3].

## Results

Hydrolyses were performed with Flavourzyme or Alcalase (E/S = 238 µL.g<sup>-1</sup> protein) in pH-stat (lines) and pH-drop (dashes) conditions.

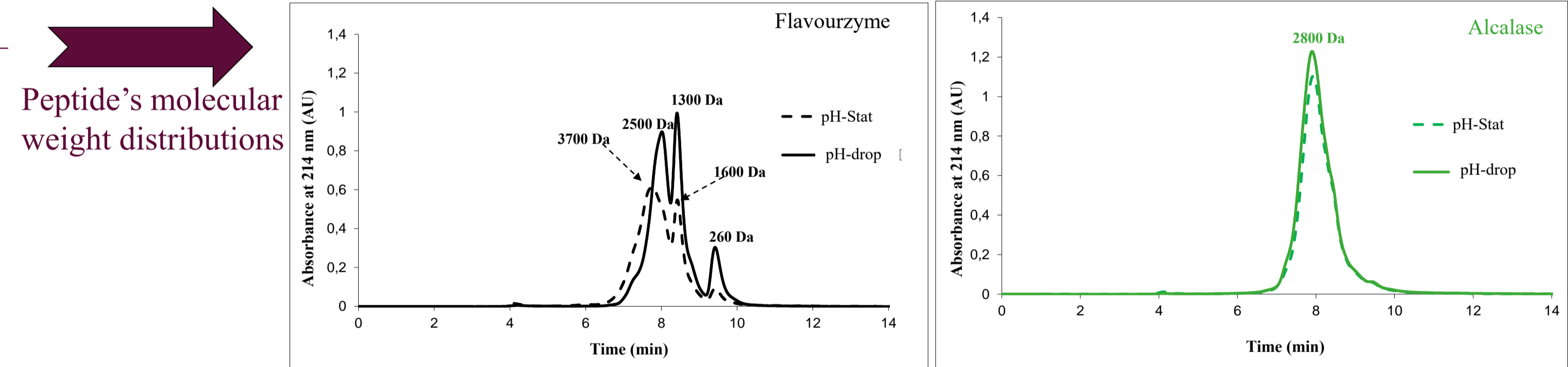
### Comparison between hydrolysis conditions pH-stat vs pH-drop

- pH-stat experiments: pH was maintained at 8.0 and DH was calculated
- pH-drop experiments: pH was only recorded



- In **pH-drop** conditions:
  - Both AL and FL can hydrolyse PPI efficiently
  - AL is 1.3 times more efficient than FL for PPI hydrolysis
- In **pH-stat** conditions:
  - AL is 1.7 times more efficient than FL for PPI hydrolysis
  - a plateau is reached at the end of the hydrolysis.
- Lack of substrate? → NO, the enzyme is saturated
- Autolysis of the enzyme? → NO, control experiments
- Lack of enzyme during hydrolysis? → Probably YES

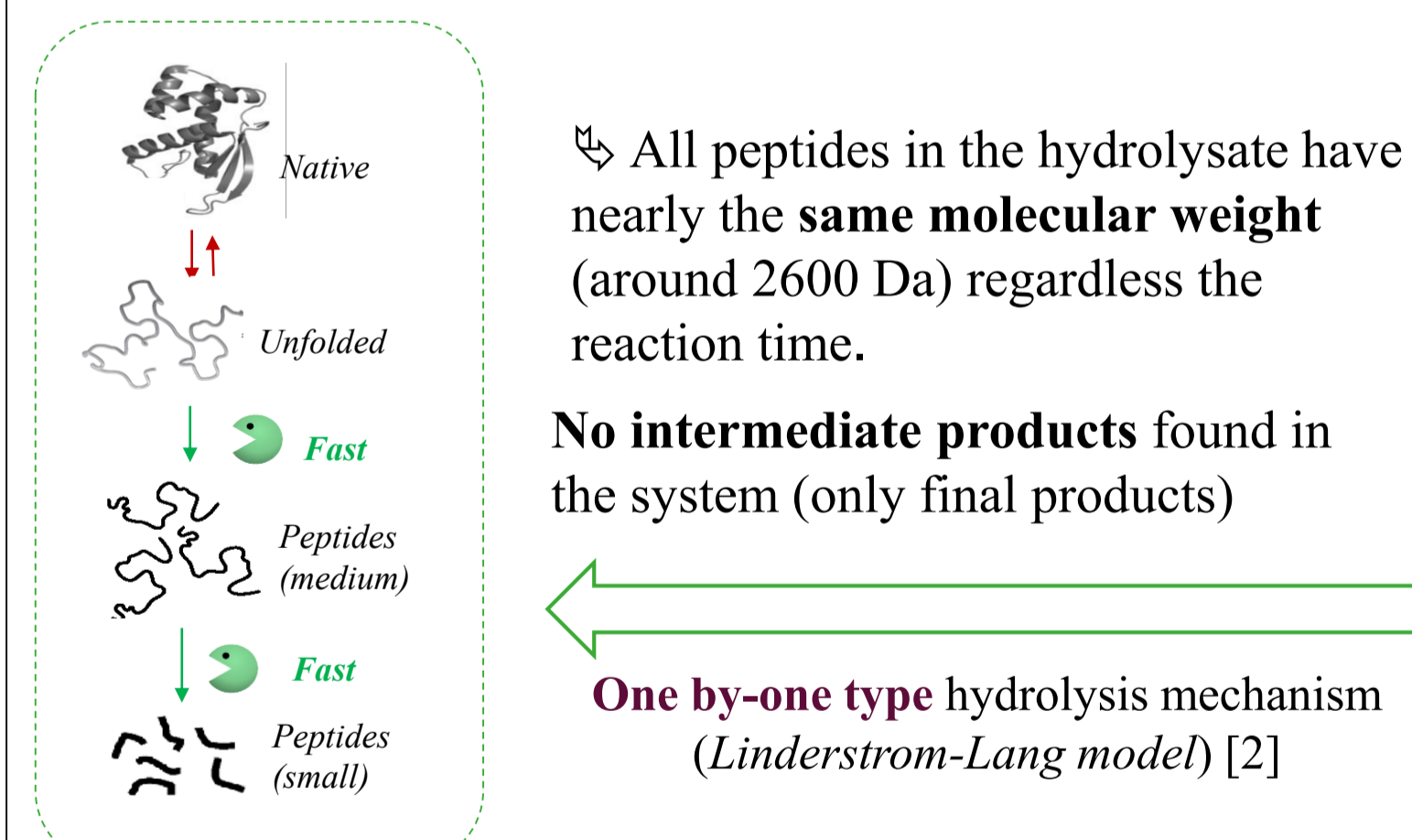
Two-hours hydrolyses were performed with Flavourzyme or Alcalase (E/S = 238 µL.g<sup>-1</sup> protein) in **pH-drop** conditions.



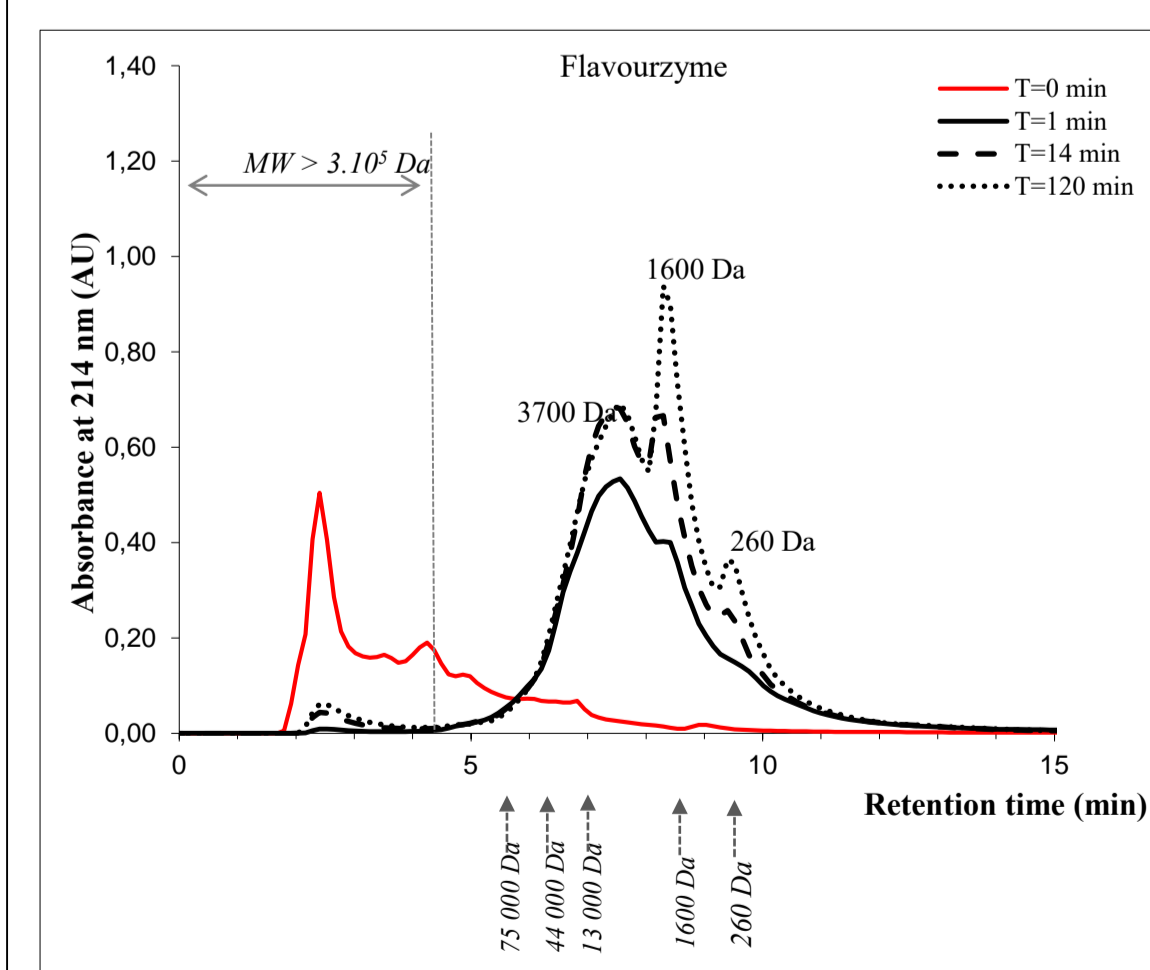
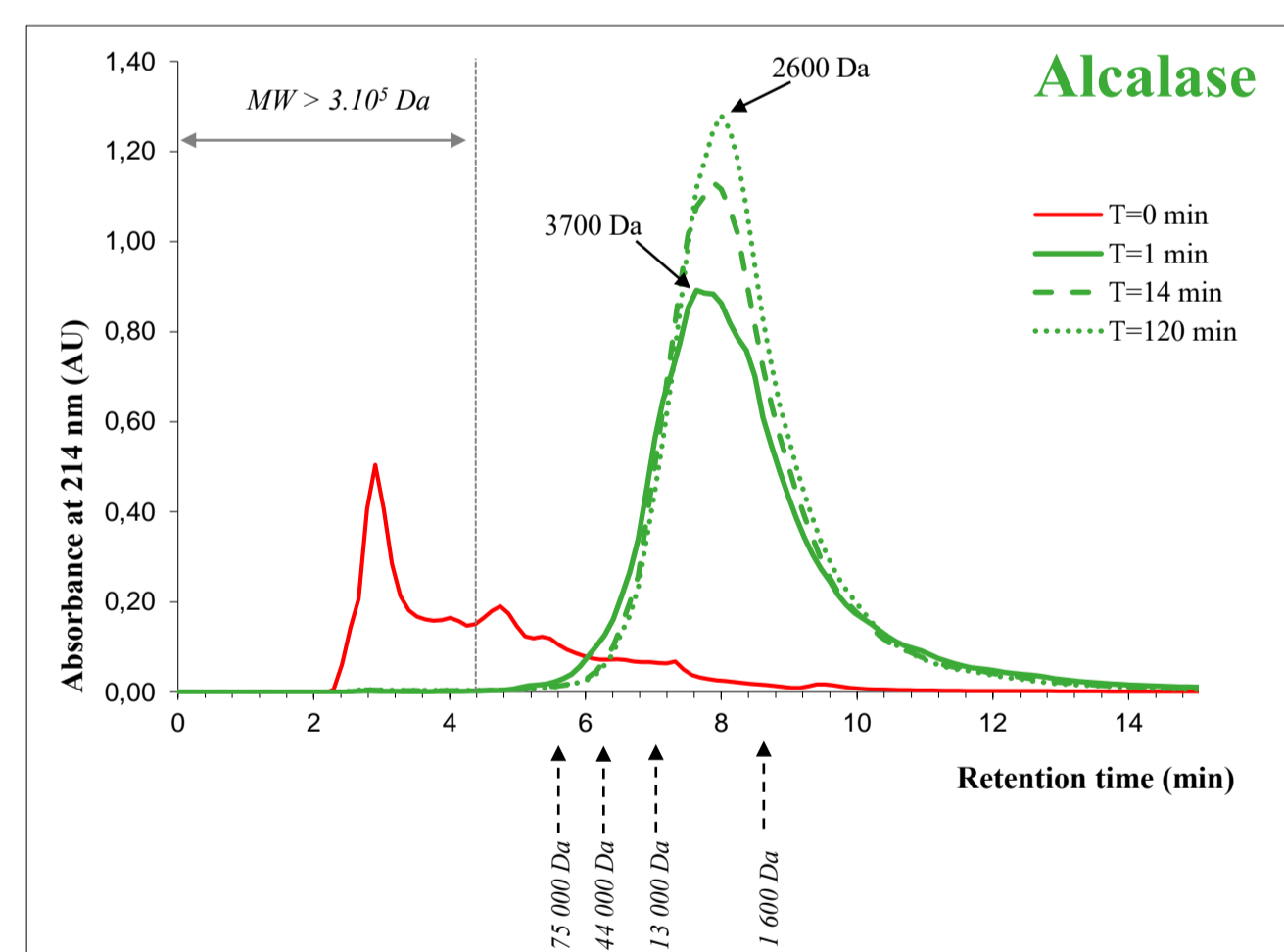
Peptide's molecular weight distributions

- Hydrolysis rates are **higher** in pH-drop conditions compared to pH-stat conditions
- For FL: can be related to its optimal pH (in pH-stat conditions, only 60% of FL activity is expressed)
- For AL: less difference in rates (between pH 6 and 8, 90% of AL activity is expressed)
- More diversity in the peptide's molecular weight when hydrolysis is conducted with FL than AL

### Hydrolysis mechanisms investigation

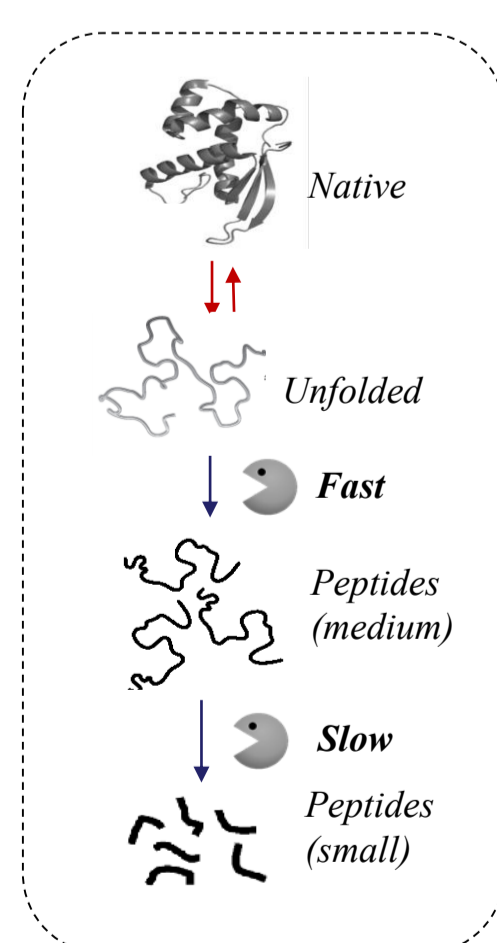


All peptides in the hydrolysate have nearly the same molecular weight (around 2600 Da) regardless the reaction time.  
No intermediate products found in the system (only final products)  
One by-one type hydrolysis mechanism (Linderstrom-Lang model) [2]

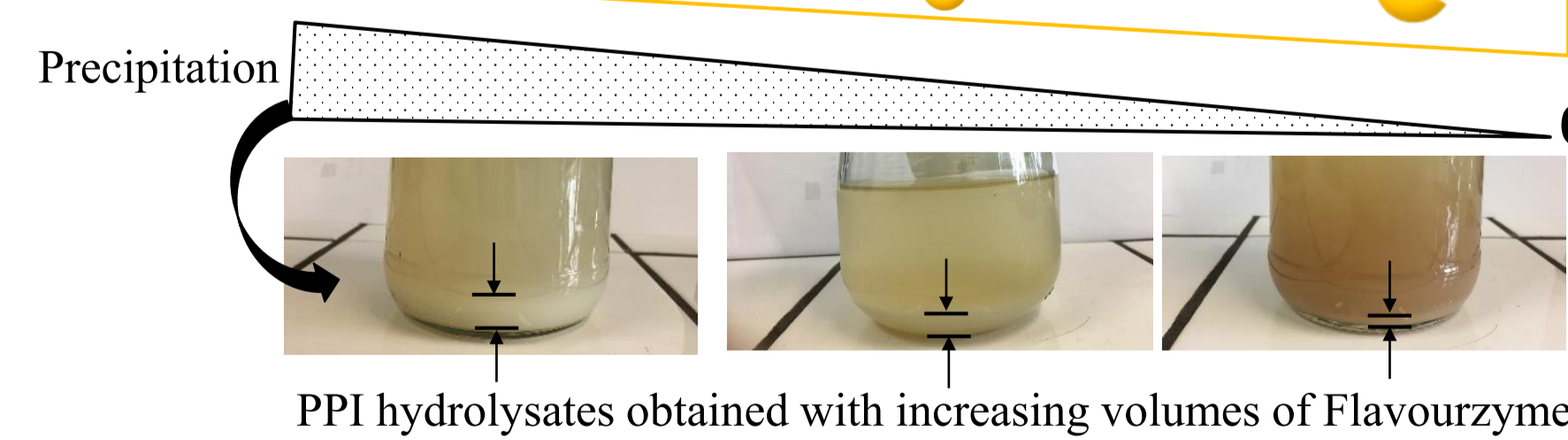
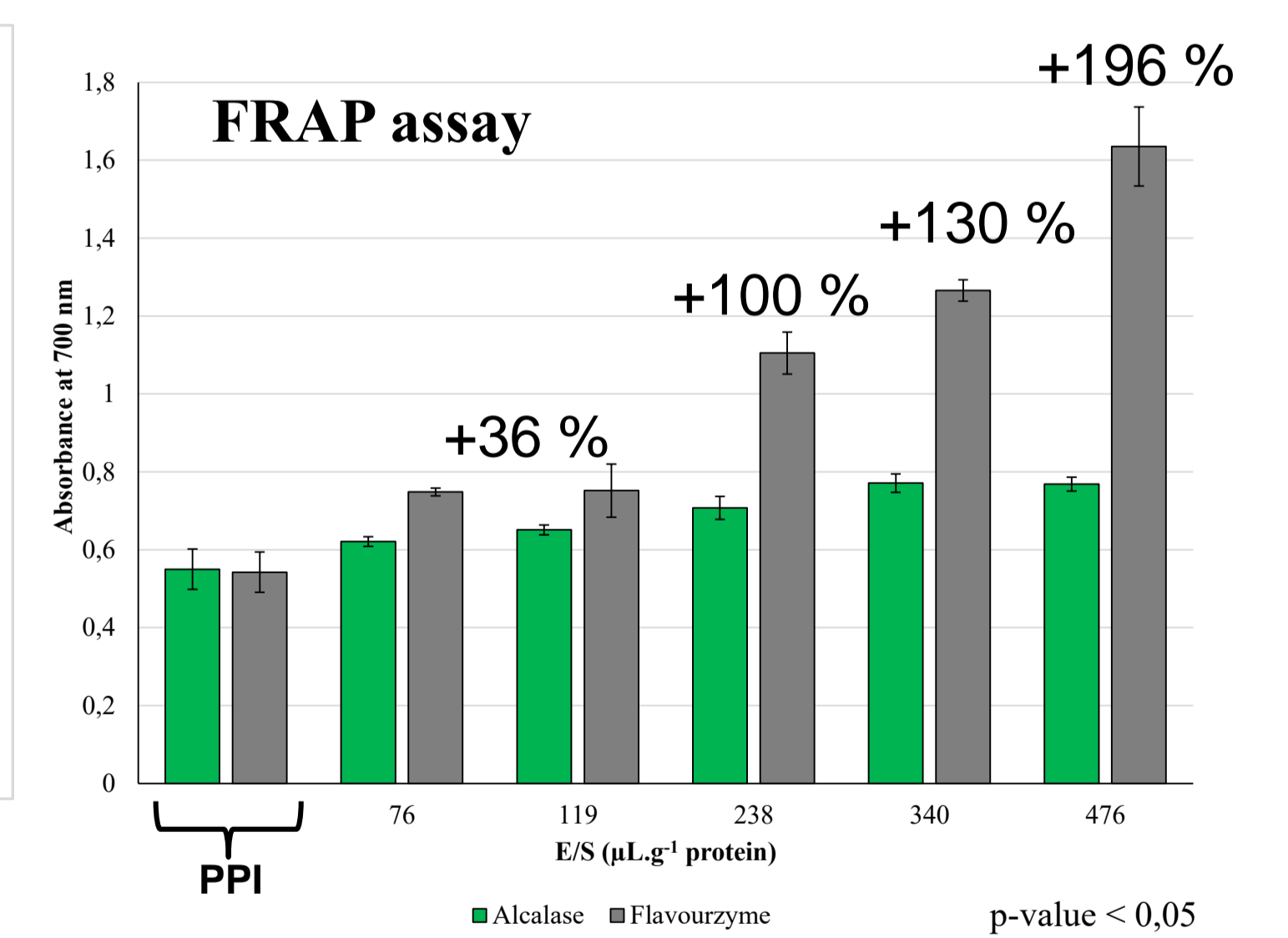
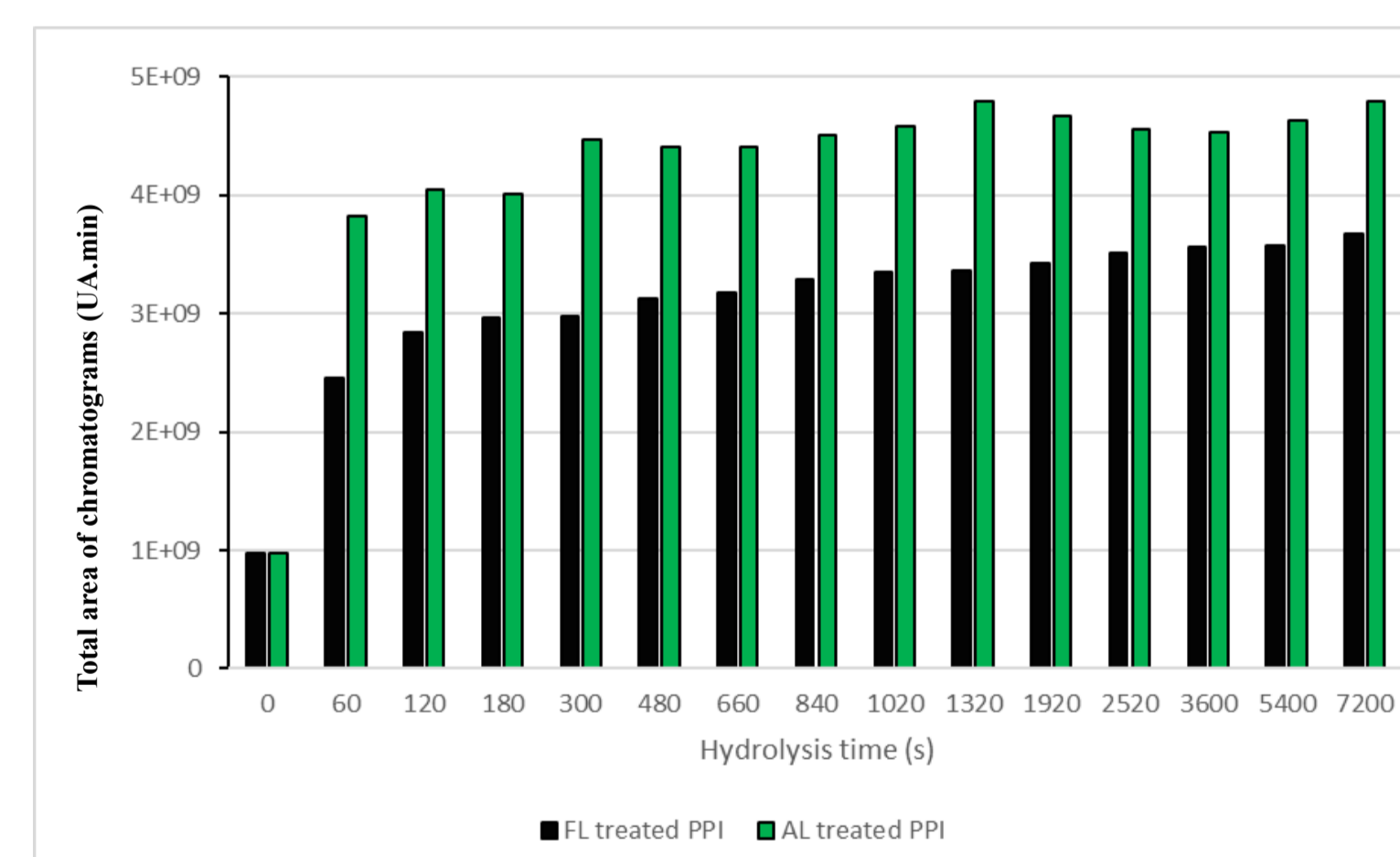


- Diversity in the MW of the peptides produced by FL (260 to 3,700 Da).
- 1 min: peptides of 3,700 Da mainly generated.
- Longer hydrolysis time: peptides of 1,600 and 260 Da produced from the intermediate peptides.

Zipper type hydrolysis mechanism (Linderstrom-Lang model) [2]



### Effect of hydrolysis on functional and bioactive properties of pea protein



Both AL and FL action increase protein solubility

- Pea protein isolate has a ferric reducing power.
- AL action only slightly improves PPI reducing power
- FL action highly improves the reducing power

## Conclusions and perspectives

PPI hydrolysis by Alcalase generates peptides of homogeneous size whereas hydrolysis with Flavourzyme generates peptides with heterogeneous sizes (260 to 3,700 Da). This allows us to conclude that Alcalase and Flavourzyme hydrolysis mechanisms are different in the conditions used in this study (E/S = 238 µL.g<sup>-1</sup> protein, pH 8.0, 50 ° C): one by one for Alcalase and zipper for Flavourzyme. Both enzymes are more efficient in pH-drop conditions so the hydrolysis reaction can be implemented in more eco-friendly conditions. Both enzymes increase protein solubility (by size-reduction) but Alcalase is more efficient than Flavourzyme. Flavourzyme generates peptides with higher reducing power values. These results show that the choice of protease is important depending on the targeted properties / applications.

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