



## Research paper

## Interaction between trypsin and alginate: An ITC and DLS approach to the formation of insoluble complexes



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## ABSTRACT

Trypsin is a protease widely used in several industrial areas for leather and meat softening and to produce enzymatic detergents, among others applications. The high demand for this enzyme has motivated the development of purification, stabilization and immobilization methods. Formation of insoluble complexes between proteins and polyelectrolytes is a methodology that may include these features. The aim of this paper is to give evidence for a novel methodology for the precipitation of the insoluble trypsin-alginate complex and hydrophobic interaction chromatography. This methodology allows the interaction between trypsin and alginate and their separation when necessary. It could be applied to isolation, stabilization and/or immobilization of trypsin. Isothermal titration calorimetry experiments showed that 232  $\mu\text{mol}$  of trypsin interacts electrostatically with 1 g of alginate to form an insoluble complex that can be separated from soluble contaminants by decantation. Dynamic light scattering experiments confirmed the calorimetric results and allowed measuring the  $R_h$  of the soluble complex at pH 3.5 (185 nm). When the optimal conditions were applied to precipitate commercially available trypsin, the recovery of the precipitation was around 92%. Finally, hydrophobic interaction chromatography allowed separating alginate from trypsin in order to obtain a polymer-free enzyme.

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## 1. Introduction

Trypsin (TRP) is a globular, monomeric serin protease produced in the pancreas of mammals as a zymogen and then secreted into the small intestine, where the enzyme enterokinase activates it by proteolytic cleavage. Its MW is 23.3 kDa and its isoelectric point is around 11 (Transue et al. [1]). This enzyme is widely used for leather softening and production of detergents. Also, in the food industry is used for meat softening in order to improve its solubility and digestibility, clarification of beer and for reducing the allergenic potential of baby food (Margot et al. [2]; Rocha et al. [3]). In the pharmaceutical industry, TRP is used for the treatment of skin wounds.

Precipitation of insoluble protein-polyelectrolyte complexes is a very useful technique to isolate, concentrate, immobilize and sta-

bilize a protein (Arroyo [4]; Alemzadeh et al. [5]; Siva Sai Kumar et al. [6]).

The advantages of using these complexes are: eco-friendly process, high-speed technique, easy scaling-up, simple equipments, and low-cost reagents.

The formation of an insoluble protein-polyelectrolyte complex depends on pH, ionic strength, temperature, charge density of both molecules and protein/polyelectrolyte ratio (Weinbreck et al. [7]; Porfiri et al. [8]; Fabian et al. [9]). Knowing the optimal conditions for the formation of the insoluble complex is very important for yielding high recoveries.

Alginate (ALG) is a linear anionic polysaccharide widely distributed in the cell walls of brown algae forming an insoluble complex with calcium and magnesium ions. This polyelectrolyte consists of two C5 epimer repeating units, (1,4)- $\alpha$ -L-gulonate (G) and (1,4)- $\beta$ -D-mannuronate (M), which are arranged in either MM, GG or alternating GM blocks. The amount and sequence of these blocks determine the properties and behaviors of the polymer. Monovalent salts of alginate are soluble in water, while divalent

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cations, such as  $\text{Ca}^{2+}$  and  $\text{Sr}^{2+}$ , interact with the functional groups of the zig-zag G blocks to form an insoluble salt.

Since ALG is a non-toxic eco-friendly polymer, it has many applications in the food industry as thickening and stabilizing agent for beers, juices and gravies. Also, it is used in pharmacology to encapsulate drugs (Chen et al. [10]) and as a thickening agent for lotions.

This paper gives evidence for the interaction between trypsin and alginate to form an insoluble complex. Combining precipitation of the insoluble TRP-ALG complex with hydrophobic interaction chromatography (HIC) could be used as a strategy to isolate, concentrate and/or immobilize TRP.

HIC is a versatile liquid chromatographic technique commonly used to separate proteins according to their hydrophobicity (Kepka et al. [11]; Guo et al. [12]). It is based on the retention of this biomolecules on weakly hydrophobic matrices in the presence of high ionic strength. In this way, HIC is a suitable technique to apply after precipitating the TRP-ALG complex, since the precipitate can be redissolved using a high concentration of NaCl without altering the tertiary structure of the enzyme. It can be used to separate it from the ALG in order to reuse the last one and to obtain a polymer-free enzyme.

## 2. Materials and methods

### 2.1. Chemical

Trypsin from porcine pancreas and the polymer alginate were purchased from Sigma Chem. Co. (USA). Sodium citrate and TRIS-HCl buffer solutions of different pH were prepared at a concentration of 50 mM. The pH was adjusted with NaOH or HCl in each case. Stock solutions of ALG 3.0% (w/v) and 1 mM TRP pH 3.0 were prepared in adequate buffer and the pH adjusted using HCl.

### 2.2. Isothermal titration calorimetry (ITC)

Three systems containing 0.1 mM TRP were prepared using different buffers: 100 mM citrate/TRIS pH 3.5; 100 mM citrate/TRIS pH 3.5 NaCl 1.0 M and 100 mM citrate/TRIS pH 8.0. These systems were titrated with 5  $\mu\text{L}$  aliquot of an ALG 0.5% (w/v) solution every 5 min. After each addition, the heat consumed or liberated was measured. All measurements were performed at 25 °C by using a VP-ITC titration calorimeter (MicroCal Inc. USA).

Finally,  $\Delta H$  was calculated and plotted vs TRP/ALG ratio. The resulting data set was fitted using MicroCal ORIGIN 7.0 software to a mathematical equation derived from the *n independent and equivalent sites* model (Jha et al. [13]). The intrinsic molar enthalpy change for the binding ( $\Delta H_b$ ), the binding stoichiometry (*n*), and the intrinsic binding constant (*K*) were obtained.

The intrinsic molar free energy change ( $\Delta G^\circ$ ) and the intrinsic molar entropy change ( $\Delta S^\circ$ ) for the binding reaction were calculated by the fundamental thermodynamic Eqs. (1) and (2):

$$\Delta G^\circ = -RT \ln K \quad (1)$$

$$\Delta S^\circ = \frac{\Delta H^\circ - \Delta G^\circ}{T} \quad (2)$$

### 2.3. Dynamic light scattering (DLS)

Various systems containing ALG were prepared in the absence and presence of TRP (TRP/ALG ratio = 0.22 mmol TRP/gr ALG), using different buffers: 100 mM citrate/TRIS pH 3.5; 100 mM citrate/TRIS pH 3.5 NaCl 1 M and 100 mM citrate/TRIS pH 8.0. The hydrodynamic ratio  $R_h$  of the particles presented in each system was measured using an ALV/CGS-3 equipped with a 22 mW He-Ne laser (632.8 nm) as light source; a scattering angle of 90° and a temperature of 25 °C.

The viscosity and refractive index was 0.89222 cP y 1.332, respectively. The matching liquid for the refractive index was *cis*-decalin.

The homodyne intensity–intensity correlation function  $G(q, t)$  was used; where *q*, the amplitude of the scattering vector, is given by equation (3) (Berne et al. [14]):

$$q = \frac{4 \pi i \sin(\theta/2)}{\lambda} \quad (3)$$

For a Gaussian distribution of the intensity profile of the scattered light,  $G(q, t)$  is related to the electric field correlation function  $g(q, t)$  by Eq. (4) (Nicolai et al. [15]):

$$G(q, \tau) = B (1 + \beta |g(q, \tau)|^2) \quad (4)$$

where *B* is the experimental baseline,  $\beta$  is a constant depending on the number of coherence areas generating the signal ( $0 < \beta < 1$ ) and  $\tau$  is the decaying time.

The diffusion coefficient *D* is related to *q* and  $\tau$  through Eq. (5):

$$D = \frac{1}{q^2 \times \tau} \quad (5)$$

The hydrodynamic ratio  $R_h$  of each particle was calculated using the Einstein-Stoke Eq. (6) (Some et al. [16]):

$$D = \frac{k_b T}{6 \pi \eta R_h} \quad (6)$$

The software GENDIST (GenR 94 v. 4.0) was used to perform the inverted Laplace transform using the REPES algorithm (*Regularized Positive Exponential Sum Program*) and to determine the  $R_h$ . The standard deviation of 4 measurements was used as an estimate of the error.

### 2.4. Precipitation of TRP with ALG

Various systems containing TRP (40  $\mu\text{M}$ ) and ALG were prepared using 100 mM citrate/TRIS buffer pH 3.5 in order to obtain different ratios: 0.17; 0.22; 0.33; 0.66; 4.00 mmols TRP/gr ALG. The insoluble complex is formed after 10 min of incubation. Then, each system was centrifuged at 1370g for 10 min and the supernatant was separated from the pellet. The last one was redissolved by adding 100 mM citrate/TRIS buffer pH 8.2 NaCl 1 M. Finally, the total amount of protein present in each fraction was measured. The standard deviation of 2 measurements was used as an estimate of the error.

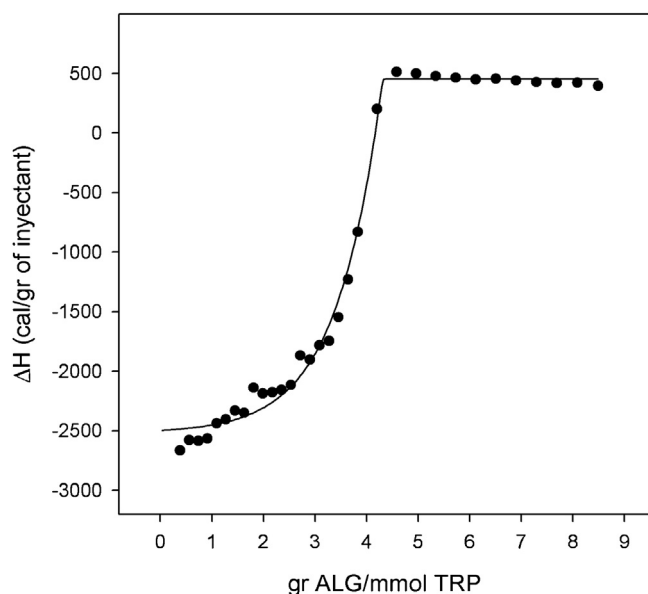
### 2.5. Hydrophobic interaction chromatography (HIC)

Experiments were performed using a FPLC System (Pharmacia Biotech) with a 1-mL Phenyl-sepharose column, at a flow rate of 0.75 mL/min and 25 °C.

Two TRP solutions were used. One solution contained TRP 2 mg/mL (no ALG) in 50 mM TRIS-HCl buffer pH 8.0 containing  $(\text{NH}_4)_2\text{SO}_4$  2.0 M; and the other, TRP 0.9 mg/mL precipitated with ALG as explained in section 2.5 and redissolved with 50 mM TRIS-HCl buffer pH 8.0 containing  $(\text{NH}_4)_2\text{SO}_4$  2.0 M.

Retention of TRP was performed adding 500  $\mu\text{L}$  of the solution to the column. Elution was performed by gradient elution of  $(\text{NH}_4)_2\text{SO}_4$  from 2.0 M to 0 M in 50 mM TRIS-HCl buffer pH 8.0.

The concentration of TRP in each fraction collected was determined by measuring its catalytic activity (Johnson et al. [17]) and absorbance at 280 nm. The catalytic activity of TRP was determined by using the chromogenic substrate *N*- $\alpha$ -benzoyl-arginine-*p*-nitroanilide (BAPNA). TRP specifically hydrolyzes this substrate rendering *p*-nitroaniline, a compound with a maximum absorption wavelength of 400 nm and a molar extinction coefficient of 18.1  $\text{mM}^{-1} \text{cm}^{-1}$ . TRP and BAPNA (0.85 mM) were mixed in 50 mM TRIS-HCl buffer pH 8.0, the absorbance at 400 nm was



**Fig. 1.** binding-isotherm graph of the calorimetric titration of TRP (0.1 mM) with ALG 0.5% (w/v). Medium 100 mM citrate/TRIS pH 3.5. Temperature 25 °C. The experimental data were fitted to a *single set of identical sites* model.

monitored for 90 s and plotted against time. The experimental data was fitted to a simple linear regression model and the slope of the fitted line was used to calculate the units of activity of TRP. One unit of activity was defined as the amount of TRP needed to produce 1  $\mu\text{mol}$  of *p*-nitroaniline in 1 min.

The concentration of ALG was measured using the Phenyl-sulfuric acid method (Dubois et al. [18]).

### 3. Results and discussion

#### 3.1. Isothermal titration calorimetry

**Fig. 1** shows the binding-isotherm graph  $\Delta H$  vs. ALG/TRP ratio, obtained at pH 3.5. This pH was chosen from several turbidimetric experiments that shown that this is the optimal pH to form the TRP-ALG insoluble complex (data not shown).

The experimental data was fitted to a *single set of identical sites* model and the intrinsic molar enthalpy change ( $\Delta H_b$ ), the binding stoichiometry ( $n$ ), the intrinsic binding constant ( $K$ ), the intrinsic

**Table 1**

Thermodynamic and binding parameters of the TRP-ALG interaction obtained by ITC at pH 3.5 (Error: SD,  $n = 3$ ).

$n/n$ (molar ratio) [g ALG/mmol TRP]	$4.31 \pm 0.05$
$k$ (affinity constant) [ $\text{M}^{-1}$ ]	$2.8 \cdot 10^3 \pm 4 \cdot 10^4$
$\Delta H^\circ$ (cal/g)	$-2970 \pm 50$
$\Delta S^\circ$ (cal/g/K)	-26
$\Delta G^\circ$ (cal/g)	-4722

molar free energy change ( $\Delta G^\circ$ ) and the intrinsic molar entropy change ( $\Delta S^\circ$ ) were calculated (**Table 1**).

It can be seen that  $\Delta H_b$  was negative indicating that the interaction between TRP and ALG is an exothermic reaction and therefore releases heat to the medium. Also,  $\Delta S^\circ$  was negative indicating that the disorder of the system decreases because of the formation of the complex. The negative values of these thermodynamic parameters confirm that the interaction between TRP and ALG is purely electrostatic and it is consistent with the high effect of pH and ionic strength on the solubility of the complex.

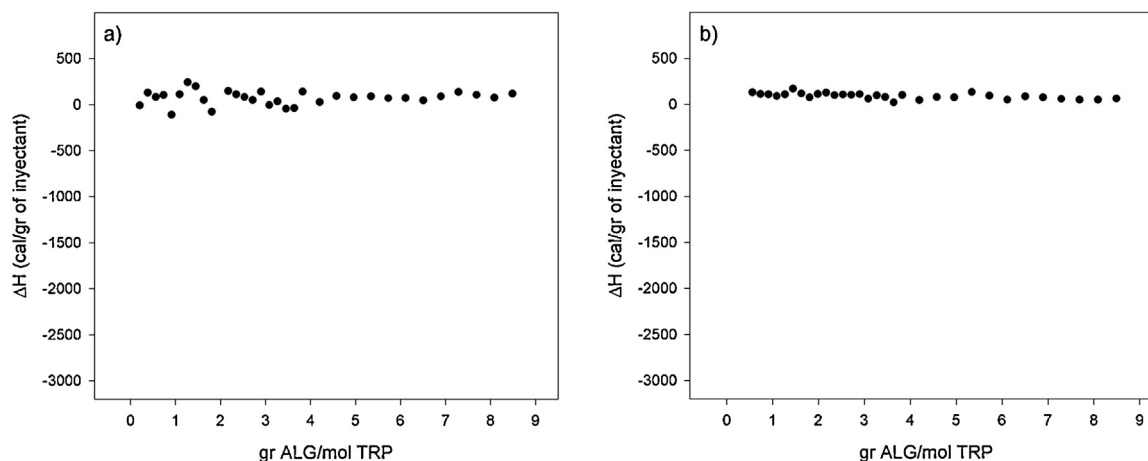
**Fig. 2a** and **b** shows the binding-isotherm graph obtained using 100 mM citrate/TRIS buffer pH 3.5 containing NaCl 1.0 M and 100 mM citrate/TRIS buffer pH 8.0, respectively.

It can be seen that both systems presented constant values of  $\Delta H$  close to zero along the experiment. This indicates that TRP and ALG do not interact in these conditions. In the first case, high ionic strength inhibits the electrostatic interaction between the two while in the second case; the enzyme possesses low positive-charge density and cannot form the insoluble (or even soluble) complex with ALG.

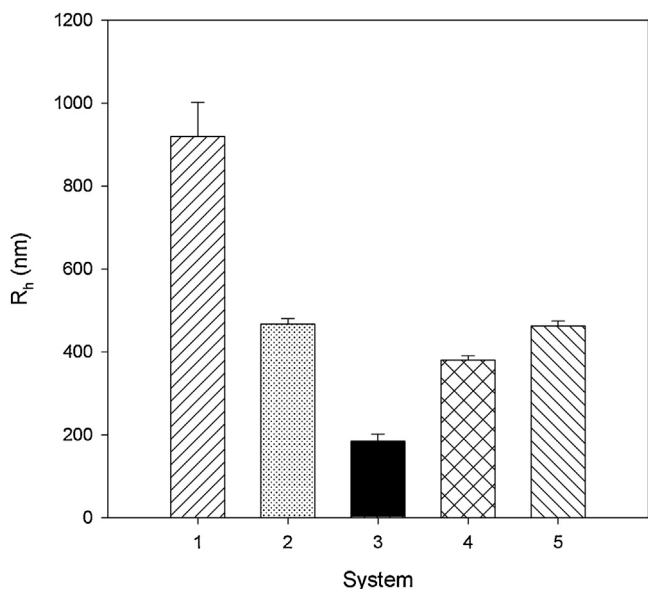
#### 3.2. Dynamic light scattering

**Fig. 3** shows the values of  $R_h$  obtained when ALG was incubated in the absence and presence of TRP in different conditions of the medium. It can be seen that when ALG was incubated at pH 3.5 in presence of TRP,  $R_h$  decreased from 920 nm (absence of TRP) to 185 nm. This indicates that the TRP-ALG complex (presenting an  $R_h$  of 185 nm) is formed at pH 3.5, corroborating the results of the ITC experiment. If NaCl was added to the medium, then the  $R_h$  was 380 nm. Since ITC experiments confirmed that TRP and ALG do not interact in presence of high ionic strength, it would be expected that  $R_h$  was 920 nm. Obviously, the structure of the ALG molecule in the absence and presence of NaCl 1 M is different.

Finally, when ALG was incubated at pH 8.0 in the absence and presence of TRP, the  $R_h$  were 467 nm and 462 nm, respectively. This



**Fig. 2.** binding-isotherm graph of the calorimetric titration of TRP (0.1 mM) with ALG 0.5% (w/v). Medium 100 mM citrate/TRIS (a) pH 3.5 NaCl 1.0 M and (b) pH 8.0, respectively. Temperature 25 °C.



**Fig. 3.** Hydrodynamic ratio ( $R_h$ ) of different systems: (1) ALG pH 3.5; (2) ALG pH 8.0; (3) ALG-TRP pH 3.5; (4) ALG-TRP pH 3.5 NaCl 1.0 M; (5) ALG-TRP pH 8.0. Error: SD,  $n=4$ . Medium 100 mM citrate/TRIS. Temperature 25 °C.

indicates that no complex is formed at pH 8.0, corroborating the results obtained using the ITC technique.

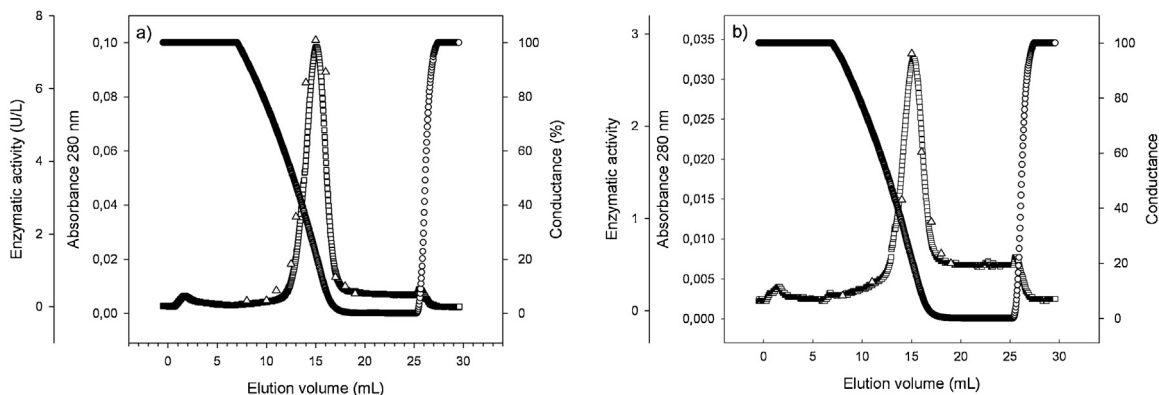
### 3.3. Precipitation of TRP with ALG

Fig. 4 shows the recovery of TRP by precipitation with different concentrations of ALG (i.e., different TRP/ALG ratios). It can be seen that 92% of the total TRP (measured by absorbance at 280 nm) was recovered in the precipitate when working with a TRP/ALG ratio of 0.22 mmols TRP/gr ALG. The recovery calculated by catalytic activity was around 97%. This result indicates that ALG is suitable for extracting TRP from pancreas by precipitation with high recovery and keeping the enzymatic activity.

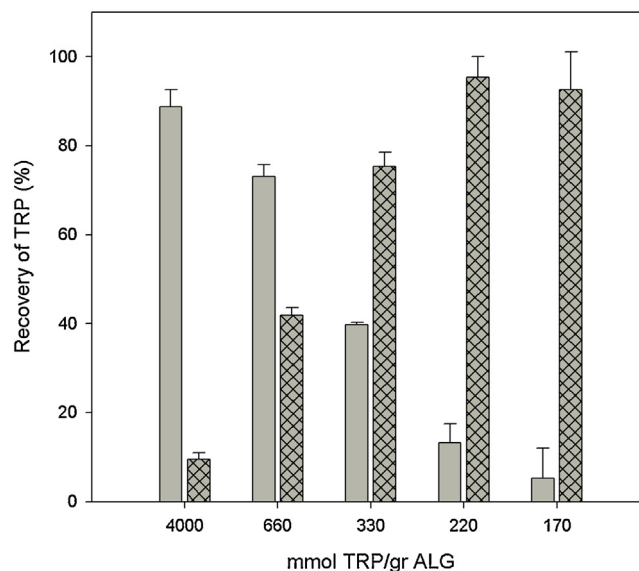
It is worth noting that TRP/ALG ratios below or above 0.22 mmols TRP/gr ALG yielded to less TRP precipitated.

### 3.4. Hydrophobic interaction chromatography

Fig. 5a and b shows the chromatogram of TRP in the absence of ALG and TRP precipitated with ALG and redissolved, respectively. It can be seen that in both cases TRP was eluted at around 15 mL with



**Fig. 5.** chromatogram of TRP in the (a) absence of ALG and (b) TRP precipitated with ALG and redissolved. A FPLC System (Pharmacia Biotech) was used with a 1-mL Phenyl-sepharose column, at a flow rate of 0.75 mL/min and 25 °C.



**Fig. 4.** Recovery of trypsin after precipitating with different concentrations of ALG. (crossed pattern) precipitate; (grey) supernatant. Error: SD,  $n=2$ . Medium 100 mM citrate/TRIS buffer pH 3.5.

**Table 2**

concentration of alginate before and after HIC.

System	Abs 490 nm	[ALG] (%P/P)
ALG 0,017% P/P	0.653	0.015
Redissolved precipitate	0.214	0.005
Supernatant	0.382	0.009
Fractions 13–16	0.001–0.003	–

peak widths of 6.17 mL and 5.63 mL, respectively. This indicates that the elution of TRP is not modified by the presence of ALG.

Also, the concentration of ALG was measured in fractions 13, 14, 15 and 16. The results are summarized in Table 2. It can be seen that ALG did not elute in the fractions were TRP did. This is very promising since precipitation and HIC could be used together to obtain polymer-free TRP.

## 4. Conclusion

Trypsin is a widely used enzyme and it is necessary to develop isolation, stabilization and immobilization methods that ensure not only high purification and/or recovery, but also low costs, use of simple equipment and eco-friendly reagents, and the possibility to

operate at a large scale. Many methods were developed in order to produce, isolate and stabilize trypsin. Some of them include many chromatographic steps, including affinity chromatography, resulting in high-cost, low-yield methods (Bougatef et al. [19]; Khantaphant et al. [20]; Jellouli et al. [21]). Even more, some methods use toxic compounds and so, the correct disposal of waste is needed, increasing costs (Schneider et al. [22]). On the other hand, the production of trypsin in recombinant systems such as *Pichia pastoris* and *Escherichia coli* has low yields, due to issues related to post-translational modifications, proteolytic activity inside the cell (or early activation of trypsinogen) and refolding of inclusion bodies (Macouzet et al. [23]).

Precipitation of the insoluble trypsin-alginate complex meets all the conditions listed above: the recovery is around 97%, it is a very simple technique already employed in many isolation protocols and easy to scale up. Also, it is worth noting that alginate is an eco-friendly polymer.

On the other hand, hydrophobic interaction chromatography is not such a simple technique but it presents two advantages: separates alginate from trypsin and might increments purity when isolating trypsin from an extract. The first allows recycling and reusing the polymer while broadening the applications of trypsin. Also, hydrophobic interaction chromatography (and other chromatographic techniques) is currently used in many purification protocols which improve the prospects to use it in the purification of TRP.

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