Kinetic studies of carrier conjugated protease inhibitors

Enrique Argenis López Olvera

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Biology Education Centre and Department of Chemistry, Uppsala University
Supervisor: Gunnar Johansson
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Abstract
Conjugates of soybean trypsin inhibitor (SBTI) and potato serine protease inhibitor (PSPI) immobilized on metal oxide particles of ~100nm diameter were prepared. Inhibition of trypsin hydrolysis of BAPA by these conjugates was measured and enzyme kinetics constants $k_{\text{cat}}$, $K_M$, $k_{\text{cat}}/K_M$ and $k_i$ were determined. Metal oxide particles presented an inhibitory effect similar to that of a competitive inhibitor, noticed through the increase value of the $K_M$ constant. Furthermore, PSPI conjugates had the highest inhibition of trypsin, illustrated by the significantly higher value of $K_M$ relative to the value for particles only.

1. Background

1.1 Conjugation of biomolecules.
Biomolecules have a widespread involvement in both research and development. Fields of interest for biomolecules range from medicine to sensor technologies (Pandey et al. 2008, Spicer et al. 2018). One of the main reasons of the interest on biomolecules, particularly of proteins and enzymes, is their physical or chemical properties. For enzymes, selectivity and environmental working conditions outstanding as desirable attributes. However, many applications for this group of biomolecules require the use of harsher conditions that hamper their performance or compromise their stability. One of the approaches that has been explored for a long time to solve this issue is the insolubilization of enzymes through their immobilization on insoluble supports (Bilal et al. 2019). Immobilization of proteins is a wide field in which several techniques have been developed, employing both physical and chemical methods. Covalent attachment is a chemical method that vastly improves protein stability and allows for easy reusability/recovery of proteins. This method employs carrier or support materials with a reactive surface to form covalent bonds with the surface of proteins, either directly or through a reactive linking molecule. One significant setback on this method is the likelihood of activity loss of the immobilized proteins due to random binding to the surface of the protein. (Hermanson 2013, Bilal et al. 2019). Covalent attachment of proteins may be included within the term ‘bioconjugation’ or conjugation of biomolecules. Literature on bioconjugation is vast and includes aspects of the chemical reactions involved, the materials used and more.

1.2 Support materials
A huge variety of materials have been studied and reported before and are available for bioconjugation. Possible supports may be either organic, such as cellulose, or inorganic. The material of choice is therefore dependent on the potential application of the conjugate (Brena et al. 2013). In this sense, inorganic particles ranging from nano to microsize are very attractive for biocatalysts design or enzyme conjugates due to their physical properties and to their attributes in key factors, mainly their high surface area and high enzyme loading (Hui Zhou et al. 2005, Pandey et al. 2008). Metal oxide particles are specially interesting supports not only for biocatalysis, but also for applications in medicine. Regardless of the unique characteristics a support material may have, it must fulfill two essential criteria. It must have a reactive surface to form chemical bonds and it must be stable, this being specially important for nanosized particles which tend agglomerate and precipitate (del Pino et al. 2013). Metal oxide particles are rarely able to form covalent bonds with biomolecules and for this reason they typically must undergo surface modification to fulfill the two criteria. Several methods of surface modification are available and the method of choice is dependent on both the intended application and the properties of both the support and the biomolecule. Popular methods of surface immobilization include ligand-exchange, polymer coating and silanization (del Pino et al. 2013).
1.3 Silanization
Many of the available metal oxide supports have hydroxyl rich surfaces. This surface is useful for non-specific physical adsorption, however renders them unable to form bonds with biomolecules. Silanization is a common method of surface modification to provide various supports, including metal oxide particles, with reactive surfaces (del Pino et al. 2013). Functional organosilanes are commonly used for silanization of surfaces and their diversity allows for multiple surface properties. Multiple organosilanes are commercially available, but three are the most commonly used for silanization of particles: aminopropyltriethoxysilane (APTES), glycidoxypropyltrimethoxysilane (GPTMS) and mercaptopropyltrimethoxysilane (MPTMS) which provide the supports with exposed amino, epoxy and thiol functional groups available for bonding (Cass & Ligler 1998).

![Figure 1. Structure of APTES on the left and of GPTMS on the right.](image)

Silanization of particles is frequently done in solution in either aqueous media, in organic solvent or in organic solvent/aqueous media. The media of choice yields different silicon layers of different properties. Silanization performed on aqueous or partly aqueous media results in silicon multilayers without a specific orientation as a result of polymerization of the molecules prior to their binding to the support, as is presented in Figure 2. On the other hand, silanization on organic solvent tends to result in a silicon monolayer (Hermanson 2013). Silanization in organic solvent is better fit for specific binding of enzymes or to achieve a desired orientation.

1.4 Protein conjugation chemistry
Both epoxy and activated-amine groups react rapidly and in high yield with nucleophile functional groups, forming stable amide or secondary amine bonds. Epoxy rings may react with primary amines, sulfhydryls or hydroxyl groups under an alkaline environment in a ring opening process, as shown in Figure 3a (Hermanson 2013). Primary amines are greatly used for conjugation due to their common occurrence in biomolecules and their chemical versatility. Binding reactions for this groups follow either the acylation or the alkylation routes. Amide formation is among the most commonly used reactions for protein and peptide binding, shown in Figure 3b. It is a spontaneously reaction but it is not optimal in aqueous media. For this reason, methods adding reactive molecules like N-hydroxysuccinimide (NHS) or carbodiimides are used to improve binding rate (Spicer et al. 2018).
Figure 2. Silanization reaction under partly aqueous media. Organosilanes polymerize before binding to the support.

Figure 3. Chemistry in the conjugation reactions. a) Ring opening process in the conjugation with epoxy groups. b) Amide bond formation reaction. c) Conjugation through zero-length linker CDI.
1.5 Activation of amino functional groups

Non-specific protein binding or binding without specific orientation typically targets amino groups from lysine residues on its surface (Spicer et al. 2018). Epoxy terminated molecules such as GPTMS may bind directly to this amino groups, as presented in Figure 3a. Linker molecules are used to specifically bind biomolecules to amino terminated surfaces, to provide them with some orientation or simply to improve binding rate (Spicer et al. 2018). Carbonyldiimidazole (CDI) has been used to bind proteins to amino terminated molecules (Spicer et al. 2018). It works as a ‘zero-length linker’, adding only one atom to the conjugate. CDI will react with a nucleophile, such as the amine in APTES, and form a reactive intermediate, as shown in Figure 3c. This intermediate conjugate is stable and prone to covalently bind to amine (or other nucleophiles) containing molecules (Spicer et al. 2018). CDI has been similarly used for other applications, although activation is usually done on carboxyl terminated molecules. CDI is rapidly hydrolyzed and must be used on non-aqueous media (Hermanson 2013).

1.6 Enzyme kinetics

Enzyme kinetics refers to the rates, or speed, of chemical reactions catalyzed by enzymes. This speed is typically expressed in mathematical terms, in the form of a rate equation which describes its dependence to the concentration of reactants. The Michaelis-Menten equation, shown in eq 1, is one such fundamental equation and broadly used in enzymology. This equation was originally derived from a single substrate enzymatic reaction and assumes a steady state on the concentration of the enzyme-substrate complex (Alberty 2011, Punekar 2018a).

\[ v = \frac{V_{\text{max}} [S]}{K_M + [S]} \]  

(1)

Kinetic constants from this equation, namely \( V_{\text{max}} \) and \( K_M \), provide relevant information on the performance of enzymes. \( V_{\text{max}} \) represents the maximum velocity of the catalyzed reaction at saturating concentrations. This constant may be substituted by the turnover number (\( k_{\text{cat}} \)), as shown in eq 2, for a more general form (Punekar 2018a).

\[ V_{\text{max}} = k_{\text{cat}} [E] \]  

(2)

On the other hand, \( K_M \) may be regarded as an apparent dissociation constant for the enzyme-substrate complex, provided some assumptions. A more useful interpretation of the constant in enzymology however, is the concentration at which the reaction reaches half of its \( V_{\text{max}} \). The rate between the turnover number and the michaelis-menten constant (\( k_{\text{cat}} / K_M \)) provides a hint on the kinetic efficiency of the enzymes, which is a useful way to compare enzymes (Punekar 2018a).

A common and straightforward way to determine the value of the aforementioned kinetic constants, is through the collection of data from reaction rate under various substrate concentration. A rate (\( v \)) vs substrate concentration ([S]) plot is then built with this data, namely a saturation curve. The kinetic constants can be determined either through graphical methods, such as through the linearization of the saturation curve into a Lineweaver-Burk plot (double-reciprocal plot), or through statistical methods like non-linear regression (Punekar 2018b).

On simple method to evaluate Trypsin activity is through the measurement of Na-Benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPA) hydrolysis and the release of p-nitroaniline through spectrophotometric measurements at \( \lambda 410 \)nm, assuming standard Michaelis-Menten kinetics as reported in previous literature (Erlanger et al. 1961, Billinger & Johansson 2018).
1.7 Inhibitors on enzyme kinetics
Enzyme catalyzed reactions can be inhibited in a number of ways, like through the addition of certain molecules that block turnover of substrate. Inhibition can be done in a reversible or an irreversible way, and the former can either follow a competitive or a noncompetitive mechanism (Copeland 2000). Most of the protease inhibitors, among which trypsin inhibitors are included, display a competitive behavior. This is the case for soybean trypsin inhibitor (SBTI) and for potato serine protease inhibitor (PSPI). This inhibitors are classified in Kunitz type, which are relatively small with only around 20kDa MW and display a binding site analog to the enzyme substrate. The inhibitor proteins count 181 amino acid residues the former and 185 the latter. This pseudo-substrate inhibitors may be graphically represented in the classic lock-and-key fashion (Thomassen et al. 2004, Farady & Craik 2010, Guerra et al. 2016).

The role of inhibitory molecules is also represented in rate equations as the equilibrium constant $K_i$, for the dissociation of the enzyme-inhibitor complex. In this sense, the $K_i$ constant has a similar meaning to that of the Michaelis-Menten and is a useful tool to represent the effect of the inhibitors. Competitive inhibitors are expected to induce a increase of the $K_M$ while keeping $V_{max}$ of the reaction unchanged. It may be determined through graphical or statistical methods (Copeland 2000).

1.8 Aim of the project
The planing of this project came as the first step towards the development of a system/formulation to be used on medical applications. On such use would be to assist in the treatment of patients suffering the exogenous action of proteases or as a means to prevent further infections of various kinds (Agbowuro et al. 2018). To this end, the choice for the carrier material were zinc oxide (ZnO) and titanium oxide (TiO$_2$) particles of a size of around 100nm diameter, as this oxides have been used before for similar purposes (Agbowuro et al. 2018).

2 Methods

2.1 Protein conjugation

2.1.1 Silanization of particles
TiO$_2$ and ZnO particles (~100nm diameter) were silanized prior to the immobilization of protein. Silanization was carried out with APTES (amino functional group) and GPTMS (epoxy functional group).
Reaction was carried out at 40°C. 0,5g of metal oxide particles were weighed and placed in a 50ml tube and suspended in 10ml of ACN. The suspension was kept 15 minutes in ultrasound bath for better dispersion of the particles. 2,5ml of silane solution (APTES or GPTMS) were then added to the suspension. The suspension was kept under end-over-end mixing overnight.
The reaction was stopped by centrifugation of the particles and disposal of the ACN supernatant. Silanized particles were repeatedly washed with ethanol and one final time with acetone and then oven-dried at ~90°C.
Particles silanized with APTES were further activated for better immobilization of proteins, according to literature. 0.15g of APTES silanized particles and 0.04g of CDI were weighed and placed in a 15ml tube and suspended in 5ml of ACN. The suspension was kept under end-over-end mixing for ~2 hours at room temperature.
The reaction was stopped by centrifugation of the particles and disposal of the ACN supernatant. Activated particles were repeatedly washed with ethanol and one final time with acetone and then oven-dried at ~90°C.

2.1.2 Protein immobilization
Immobilization of proteins (Myoglobin, SBTI & PSPI) was carried out at room temperature to prevent compromising their integrity. 0,025g of GPTMS silanized or activated APTES silanized
supports were weighed and placed in 1ml microtubes and suspended in [0,2M] NAHCO$_3$ pH 7 buffer. The suspension was kept on ultrasound bath for around 15 minutes. Protein solution was then added to the suspension, for a total reaction volume of 1ml. The suspension was kept under end-over-end mixing overnight.

The reaction was then stopped with the centrifugation of the conjugates and the storage of the supernatant. The conjugates were then washed with MQ water to get rid of adsorbed protein and finally kept suspended in 1ml of MQ water. The absorbance of the supernatant was measured at $\lambda$410nm for myoglobin and at $\lambda$280nm for trypsin inhibitors to evaluate the immobilization. The absorbance measurements recorded were used to determine the concentration of protein through the Beer-Lamber law, shown in eq 3.

$$A = \varepsilon \cdot l \cdot c$$  
(eq 3)

Where $A$ stands for absorbance, $\varepsilon$ for mass attenuation coefficient ($\text{M}^{-1}\text{cm}^{-1}$), $l$ for the length of the light path (value fixed as 1cm) and $c$ for concentration ($\text{molL}^{-1}$).

Adsorption of SBTI was also evaluated. Increasing amounts of non-silanized metal oxide particles were suspended in 1ml of 39$\mu$M SBTI solution in MQ water. Suspensions were stirred for 10 minutes, then particles were precipitated by centrifugation and the absorbance of the supernatant was measured. Inhibitor concentration of the solutions was determined through equation 3.

2.2 Trypsin kinetics evaluation

Hydrolysis of BAPA by trypsin from porcine pancreas was measured through spectrophotometric measurements at $\lambda$410nm and standard Michaelis-Menten kinetics were used for its analysis. Enzyme kinetics were determined through the graphical method of the Lineweaver-Burk plot when possible and through statistical non-linear regression on the software GraphPad Prims 8. Reaction was carried out in 1ml solution containing MQ water, BAPA solution (in DMSO), trypsin solution and immobilized inhibitors, or silanized particles & free inhibitors. Concentrations used are showed in Table 1.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAPA</td>
<td>5 – 0,2</td>
<td>$\mu$M</td>
</tr>
<tr>
<td>SBTI / PSPI</td>
<td>0,057</td>
<td>$\mu$M</td>
</tr>
<tr>
<td>Oxide particles</td>
<td>0,05</td>
<td>mg/ml</td>
</tr>
<tr>
<td>Trypsin</td>
<td>3,2</td>
<td>$\mu$M</td>
</tr>
</tbody>
</table>

Enzymatic activity was measured for a length of three to ten minutes, according to practical convenience. Measurement of BAPA hydrolysis was performed in continuous mode for the reaction without particles and at single points within the time range of the reaction in all other cases. Reaction was stopped in aliquots through three different ways: heat inactivation, inhibitor excess and reaction pH adjustment through the addition of acetic acid. The aliquots were centrifuged to precipitate the suspended particles after the reaction was stopped. The absorbance of the supernatant was measured and substrate hydrolysis plots were built.

3 Results & Discussion

3.1 Protein immobilization

Myoglobin protein was first used to verify the silanization of the particles and as a reference model for the later immobilization of trypsin.
Immobilization on TiO$_2$ particles was more efficient on the epoxy-silanized particles in all three cases, as seen in Figure 4. The percentage of immobilized protein on titanium oxide decreases as the initial concentration of the solution increases as expected, going from 89 to 74% and from 80 to 62% for the epoxy and the amino functionalized particles, respectively. This shows a trend towards an enzyme loading saturation value of the particles and an acceptable concentration range to work with. Apparent immobilization on ZnO particle was measured to be close to 100% of the initial concentration in the suspension. This may suggest that the enzyme loading saturation value is much higher for this support or that there is some other binding interaction involved.

Similar measurements were performed for the immobilization of the inhibitors. Important differences to the immobilization of myoglobin were observed, particularly in the immobilization on epoxy-functionalized particles, as is noted in Figure 5. Inhibitors appear to be more efficiently immobilized on TiO$_2$ supports, as opposed to the more efficient immobilization of myoglobin on ZnO particles. The silane of choice had a significant effect on the enzyme loading on the particles. To this regard, lower immobilization was measured on both epoxy-functionalized TiO$_2$ and ZnO particles, that being 25 and 20% of the enzyme immobilized, respectively. In contrast, loading on amino-functionalized particles was as high as 89 and 75% for TiO$_2$ and ZnO particles, respectively. This different behavior may be attributed to the varying composition and availability of reactive groups on the surface of the proteins. However, a thorough study of their surfaces will be necessary to provide a definitive answer.
3.2 Adsorption of proteins

Measurement of adsorption served to discard the contribution of non-covalently bound inhibitor on the measurement of trypsin kinetics. Adsorption on non-functionalized titanium oxide particles was greater than on zinc oxide particle also non-functionalized. Adsorption of the inhibitor on titanium oxide particles reached nearly 20% of the initial concentration (39 μM) for the highest amount. This is presented in Figure 6 and a trend towards saturation of the support is appreciated. On the other hand, adsorption on zinc oxide particles was comparatively low. It is noted in Figure 6 that saturation of inhibitor loading on ZnO particles is well below 10% of the initial concentration. It is important to note that this much adsorption happens only at relatively high amounts of suspended particles. Kinetic analysis were performed under concentrations of 0.5 mg/ml, which would represent adsorption of around 0.02% of the inhibitor. This is a negligible amount and therefore no contribution of adsorption was considered for the kinetic analysis.
3.3 Trypsin kinetics

Enzyme kinetics of trypsin from porcine pancreas were measured and recorded. A saturation curve (substrate concentration vs reaction rate) was prepared through the measurement of the substrate hydrolysis, displayed in Figure 7(a). This curve was then linearized to produce a Lineweaver-burk plot to calculate the values for the turnover number ($K_{\text{Cat}}$) and the Michaelis-Menten constant ($K_M$). Following this graphical method, the value for $K_{\text{Cat}}$ and $K_M$ was determined to be 0.3092s$^{-1}$ and 0.749mM, respectively. This values serve as a reference to evaluate the inhibition of trypsin by both the inhibitors and the metal oxide particles.

Following the same method, the catalytic values for the reaction including both amino- and epoxy-silanized particles were obtained. The concerning plots are presented in Figure 8 and results can be summarized as presented in Table 2. The behavior of ZnO particles in the suspension resulted in inconsistent results and were therefore not included. Turnover number was determined to be 0.3186s$^{-1}$ and 0.3078s$^{-1}$ for amino- and epoxysilanized particles, respectively. This values are close and of the same magnitude as that for trypsin alone. The turnover number is higher than that for the reaction with TiO$_2$-A, however this can be attributed as a statistical error. Similarly, the values for the Michaelis-Menten constant are 1.2952mM and 1.5958mM. This shift to higher values in both cases provides some insight on the influence of the metal oxide particles on trypsin kinetics. An important drop in the $k_{\text{cat}}/K_M$ rate is a consequence of this shift, suggesting that the presence of this particles on their own diminish the efficiency of trypsin.
Figure 7. Trypsin kinetics. (a) Saturation curve for trypsin with BAPA as substrate. (b) Lineweaver-Burk plot for the determination of kinetic constants.

<table>
<thead>
<tr>
<th></th>
<th>Graphic</th>
<th>Statistical</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>$K_M$ (mM)</td>
</tr>
<tr>
<td>No particle</td>
<td>0.3093</td>
<td>0.7494</td>
</tr>
<tr>
<td>TiO$_2$-A</td>
<td>0.3186</td>
<td>1.2953</td>
</tr>
<tr>
<td>TiO$_2$-E</td>
<td>0.3078</td>
<td>1.5969</td>
</tr>
</tbody>
</table>

These constants were also determined through a non-linear regression statistical method. The value of the constants is changed but it remains close to those obtained through the graphic method, adding certainty to the results.
Figure 8. Graphical representation of trypsin hydrolysis of BAPA in a titanium dioxide particle suspension. (a) Saturation curve for amino-silanized particles suspension. (b) Lineweaver-Burk plot of the same. (c) Saturation curve for epoxy-silanized particles suspension. (d) Lineweaver-Burk plot of the same.

Next step was to evaluate the effect of the conjugated inhibitors on the kinetics of trypsin. Trypsin activity was only measured with inhibitors conjugated to the amino-silanized supports after considering the expected amount of immobilized inhibitor. The same graphical method used before was not adequate for this evaluation due to both technical difficulty and to the data itself. Therefore only the statistical approach was taken here. Saturation curves under four conditions were built, displayed in Figure 9.
Figure 9. Trypsin saturation curves under the influence of inhibitors. (a) BAPA hydrolysis on media with suspended aminosilanized titanium dioxide particles. (b) Reaction on media with commercially available trypsin inhibitor conjugated on the same particles. (c) Reaction on media with PSPI conjugated on the same particles. (d) Reaction on media with suspended particle and non-conjugated PSPI.

Determination of the catalytic constants was done through non-linear regression method on data analysis software *GraphPad Prism 8*. Said values are presented in Table 3.

Table 3. Catalytic constants for trypsin hydrolysis of BAPA under the influence of inhibitors.

<table>
<thead>
<tr>
<th></th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_{m}^{app}$ (mM)</th>
<th>$k_{cat}/K_{m}$ (mM$^{-1}$s$^{-1}$)</th>
<th>$K_i$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TiO2-A</td>
<td>0.0896</td>
<td>5.7370</td>
<td>0.0156</td>
<td></td>
</tr>
<tr>
<td>TiO2-A-SBTI</td>
<td>0.0542</td>
<td>4.1000</td>
<td>0.0132</td>
<td>no valid</td>
</tr>
<tr>
<td>TiO2-A-PSPI</td>
<td>0.0633</td>
<td>17.2800</td>
<td>0.0037</td>
<td>0.0283</td>
</tr>
</tbody>
</table>
A drop in the value of the turnover number was determined for both the reactions with conjugated SBTI and PSPI inhibitors. This change in $k_{cat}$ is out of the expected outcomes, based on what is known from the inhibitors. Both the curve and the data obtained for the conjugated SBTI bear greater resemblance to those from particle only data. For the measurements with conjugated PSPI the curve display a clearly different shape and the value of $K_M$ increased about 3 times. This increase is what is expected from a competitive inhibitor, however the decrease in the $k_{cat}$ was not expected.

The $k_{cat}/K_M$ rate provides some insight as well. The drop in the rate for the reaction with conjugated SBTI is very small and near negligible as opposed for the reaction with conjugated PSPI. The $k_i$ constant was mathematically determined. The $k_i$ for conjugated SBTI could not be determined due to the estimated values of $K_M$. The value of this constant for the conjugated PSPI was determined to be 0.0283\,\mu M. This would imply that the amount of PSPI employ for the measurement was well over the value of its $k_i$.

Figure 9(d) shows the plot points generated for the reaction under the influence of unconjugated PSPI inhibitor and titanium oxide particles. It is noted that the points do not align in any clear shape and therefore it was not possible to build a saturation curve and in consequence the value for the catalytic constants could not be determined.

4 Conclusions and future outlook

Trypsin hydrolysis of BAPA was measured under different conditions. A clear influence of titanium oxide particles on the catalytic efficiency of trypsin was measured and percieved in a similar way to that of a competitive inhibitor. Whether this effect is due to adsorption or other non-covalent interactions between the particles and the other molecules was not determined on this project.

Measurements for trypsin inhibition showed ambiguous results. Inhibition by conjugated SBTI was near to not noticeable and the $k_i$ constant couldn’t be mathematically determined under present conditions. Effect of conjugated PSPI was more clear and according to what is expected of an inhibitor of this kind based on available literature. The value for the $k_i$ constant seems to be consistent, although further verification is required.

The results presented here represent a qualitative assessment of the inhibition mechanics of the conjugates. It will be convenient to continue this activities to generate data for further statistical analysis and generate a reliable, quantitative assessment. Relevant work to do in the future include the verification of the type of inhibition in which this conjugates behave. This requires the measurement of the reaction under different concentrations of inhibitor.

For additional future work an improvement on the enzyme activity measurement is suggested in order to get rid of misreadings induced by the suspended particles. Improvements would include better control of particle aggregation and the possibility of using particles of smaller sizes. Use of immobilized trypsin could result in a very well standardized method for activity measurement, although recovery of trypsin would need to be independent from recovery of inhibitors. Such standardization would greatly facilitate accurate enzyme kinetic analyses.

5 Bibliography


