

Low pI Protein Immobilization Strategy: Cationization



SnAPPShots

A brief technical report
from the Corning
Applications Group

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Introduction

The Corning® Epic® System is a high-throughput screening (HTS) label-free platform that allows for the observation of direct bio-molecular interactions in a biochemical binding assay and pharmacologically more relevant cellular response to drug in a cell-based assay. It consists of a disposable standard 384-well microplate compliant with the ANSI/SBS-microplate standard, with resonant waveguide optical biosensors integrated into each well and an HTS-compatible optical reader. It eliminates issues with detection typically associated with use of labels, such as background fluorescent interference, while providing access to new biochemical and pharmacological information for drug discovery.

Epic biochemical assays are performed on amine-coupling surfaces (Cat. No. 5041 and 5046XX1). Low pI (isoelectric point) proteins are particularly difficult to immobilize on amine-coupling based surfaces. As a rule of thumb, best immobilization results are achieved at a pH slightly below the pI of a protein, while staying between pH 5-7. Consequently, for proteins with a pI below 5, it will be difficult to obtain high levels of immobilization using standard protocols. In this application note, a method is described to increase the immobilization level of low pI proteins via cationization.

To illustrate application of this method, two different low pI proteins were investigated: trypsin inhibitor (from Glycine max, 21.5 kDa, pI 4.6) and pepsin A (hog stomach, 41.3 kDa, pI 4.0). In order to enhance the immobilization of low pI proteins on amine-coupling surfaces (Cat. No. 5041 and 5046XX1), two important parameters need to be optimized:

- First, the type and amount of amine linker used can be varied. Either ethylene diamine (ED) or ethanol amine (EA) can be used. EA introduces a neutral –OH group to the protein and is therefore less likely to hamper the activity of the protein. ED on the other hand introduces charged amine groups to the protein. It will therefore increase the binding capacity of the protein to the surface, and is preferred if the protein has little or no lysine residues available for amine-coupling. The optimum amount of linker used will typically lie in the range of 5 to 20 mole equivalent, although this number can vary with each protein.
- Second, the immobilization pH needs to be optimized to ensure high immobilization levels and also to ensure the protein remains active.

Materials/Methods

Trypsin inhibitor from Glycine max (soybean), 21.5 kDa, pI 4.6 (Cat. No. T1021), trypsin from porcine pancreas, 23.8 kDa (Cat. No. T0303), pepsin porcine gastric mucosa (Pepsin A), 41.3 kDa, pI 4.0 (Cat. No. P6887), pepstatin A, 685.89 Da (Cat. No. P5318) and ethylenediamine (ED) (Cat. No. 240729) were purchased from Sigma-Aldrich. Zeba desalting column 0.5mL (Cat. No. 89883), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (Cat. No. 22981) and sulfo-N-Hydroxysulfosuccinimide (sNHS) (Cat. No. 24510) were purchased from Pierce. 20 mM sodium acetate buffer pH 4.5, 5 and 5.5 and 10 mM HEPES buffer pH 7.4 were prepared using standard protocols.

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Epic[®]
system

Protein Cationization

Stock solutions of ED and EA were prepared at 0.5 M in water. Working ED and EA solutions are prepared by dilution 100x in 10 mM HEPES buffer pH 7.4 for the trypsin inhibitor and 10x in 20 mM sodium acetate buffer pH 4.5 for the pepsin A. Cationization was performed by adding 10 μ L of EA or ED working solution to 100 μ L of 1 mg/mL protein and incubated 90 minutes at room temperature. The protein was subsequently recovered using a desalting column.

Immobilization

User activated plates (Cat. No. 5046XX1) were used for pepsin and were activated with 10 μ L of 20 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC)/5 mM sulfo-N-Hydroxysulfosuccinimide (sNHS) (Pierce Cat. No. 22981/ Pierce Cat. No. 24510) and incubated for 30 minutes at room temperature. The plates were subsequently washed 3 times with 25 μ L H₂O with a 384-channel pipettor and centrifuged upside down (800 g/15 seconds) to ensure proper dryness of the plate.

Protein immobilization was accomplished by applying 10 μ L of protein (modified or native) at 100 μ g/mL in 20 mM sodium acetate at the required pH (4.5, 5 or 5.5) using a 384-channel pipettor (CyBi-Well) and incubated overnight at 4°C. The plates were subsequently washed three times with assay buffer, and finally, 15 μ L assay buffer was added per well.

Binding Assay

The binding assay was run on an Epic® instrument. After 25 min thermal equilibration, a baseline read was taken on the Epic plates with protein immobilized in assay buffer.

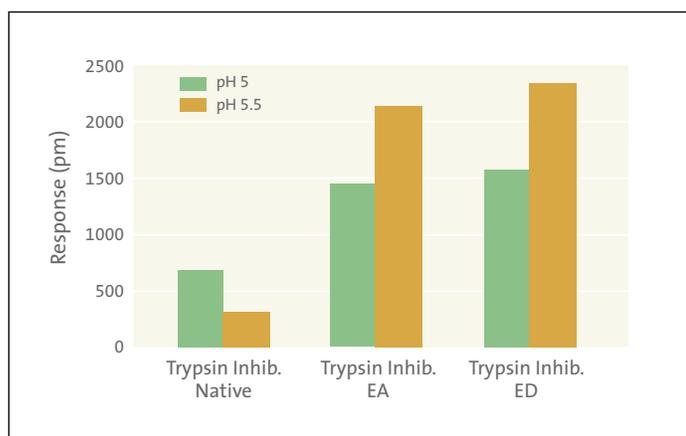


Figure 1. Trypsin inhibitor immobilization at pH 5 and 5.5 in 20 mM sodium acetate buffer. Cationization of the protein with ethanolamine (EA) and ethylenediamine (ED) as described in materials and methods.

In the next step, 15 μ L of compound were added to the plates and then mixed 10 times. The final read was taken after 25 min. thermal equilibration.

Results

Example 1: Trypsin inhibitor

Trypsin inhibitor an important protein present in many vegetable species and in particular, the soybean. Trypsin inhibitor is known as a potent anticancer compound (for review, see [1]).

This protein of 21.5 kDa has a pI value of 4.6. It is an excellent candidate for optimizing the protocol for immobilizing new and interesting low pI protein targets.

The ability of the trypsin inhibitor protein to immobilize on the amine-coupling surface was tested in sodium acetate buffer at 2 pH values: 5 and 5.5. This buffer was chosen as it is a standard buffer for Epic assays. In parallel, the ED and EA cationized protein were immobilized in the same conditions. Figure 1 shows that cationization of the protein enhanced its ability to be immobilized on the amine-coupling surface.

Next, the ability of the trypsin inhibitor to bind to its natural ligand trypsin was evaluated. Increasing concentrations of trypsin were incubated with the immobilized proteins. The results demonstrated that binding of the enzyme to its natural immobilized ligand was specific and saturable. (Fig. 2).

For the native trypsin inhibitor immobilized at pH 5.5, no signal could be observed. Only the cationized protein provided high quality data allowing determination of the binding constants. The affinity of the cationized protein to trypsin (42 nM), is in agreement with literature values (2) proving that the trypsin inhibitor activity was not altered by cationization.

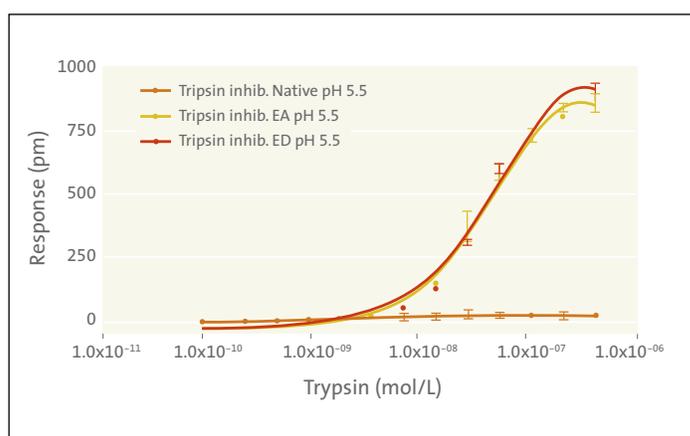


Figure 2. Trypsin inhibitor/trypsin dose response binding curves of protein immobilized at pH 5.5. Cationization of the protein with ethanolamine (EA) and ethylenediamine (ED) as described in materials and methods.

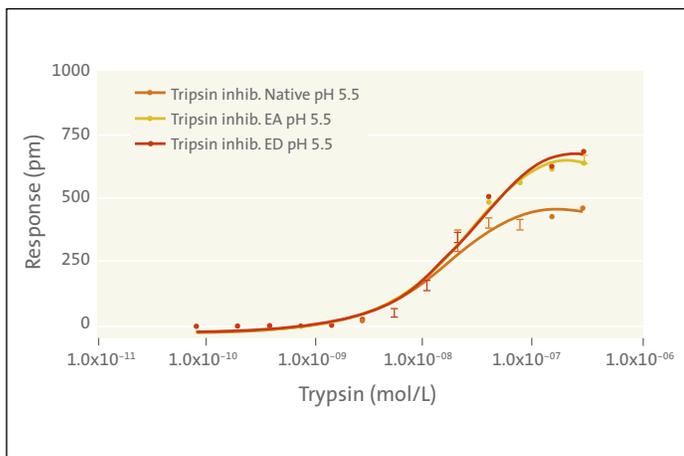


Figure 3. Trypsin inhibitor/trypsin dose response binding curves of protein immobilized at pH 5. Cationization of the protein with ethanolamine (EA) and ethylenediamine (ED) as described in materials and methods.

Alternatively, the assay can be carried out with the native protein at pH 5 (Fig. 3). Even though the immobilization was only 600 pm, trypsin binding could be observed with similar affinity.

The decision to cationize the protein, therefore depends on the size of the ligand studied and the desired pH for immobilization. Small molecule binding studies would generally require immobilization levels >2000 pm. Furthermore, certain proteins will not tolerate low immobilization pH levels.

Example 2: Pepsin A

Pepsin A is an important protease involved in the digestion of protein. It is active inside the stomach. The optimal activity for this protease is observed at pH 2 (3). This protein has a pI of 4.0 and is therefore an excellent candidate for the low pI protein immobilization protocol. In order to characterize the modified protein, a ligand binding assay was performed with a very well characterized inhibitor, pepstatin (685.89 Da).

In order to verify the integrity of the protein upon cationization, the activity of the cationized protein was studied in solution. Bovine serum albumin was immobilized on an Epic microplate (Cat. No. 5041) to which pepsin was subsequently added, either alone or in the presence of increasing concentrations of pepstatin. (Fig. 4). The inhibition efficiency is high for the native and for the cationized protein with an EC_{50} ~10 nM compared to 2 nM published earlier (4). This difference can be explained by the pH of the test at 4.5 instead of pH 2, the optimal pH for pepsin activity. Importantly, however, no significant difference in inhibition constant was found between the native and the cationized proteins.

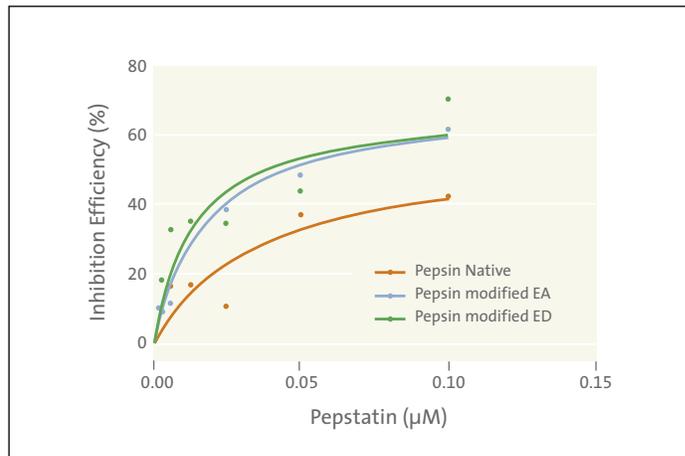


Figure 4. Pepstatin inhibition on pepsin activity measured on Epic® functional assay. Cationization of the protein with ethanolamine (EA) and ethylenediamine (ED) as described in materials and methods.

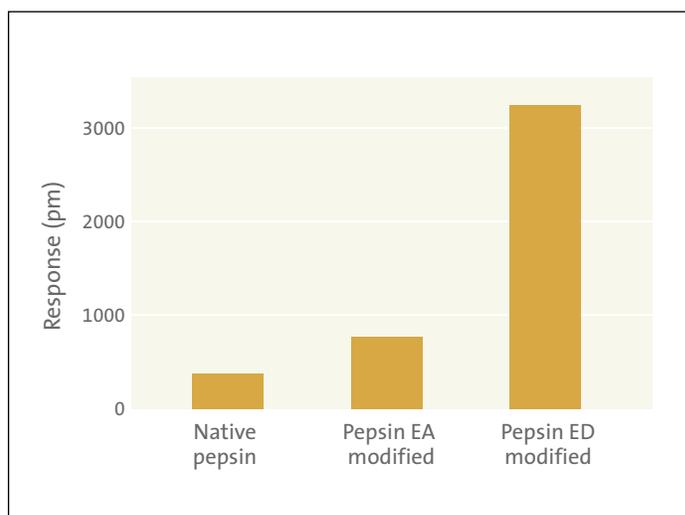


Figure 5. Pepsin (100 µg/mL) immobilization levels in 20 mM sodium acetate buffer pH 4.5. Cationization of the protein with ethanolamine (EA) and ethylenediamine (ED) as described in materials and methods.

Once the integrity of the cationized protein was confirmed, the immobilization was carried out using the standard protocols. The immobilization efficiency of native pepsin, and pepsin modified with EA and ED at pH 4.5 was carried out. The results, illustrated in Fig. 5 clearly show that ED cationized protein was immobilized to more than 3000 pm, while the native protein was immobilized to less than 400 pm.

Furthermore, Fig. 6 demonstrates that the immobilized protein cationized with ED showed specific and saturable binding to pepstatin with a measured affinity of 5×10^{-8} M. This is in the range of the inhibition constant obtained in solution with the functional Epic® assay. It is also within one log order of magnitude from published data (4).

Conclusions

- ▶ The Epic System enables label-free direct biochemical assay for small molecules and protein-protein binding to an immobilized target without the needs of labels.
- ▶ This application note illustrates that protein cationization can be used to study low pI proteins in an Epic biochemical assay.
- ▶ Modification of the protein enables immobilization onto usual surfaces using regular protocols whilst protein binding ability is preserved.
- ▶ This method offers an alternative approach to investigate difficult protein targets having low pI values for label-free biochemical binding assays.

References

1. Kennedy A.R. The Bowman-Birk inhibitor from soybeans as an anticarcinogenic agent. *Am. J. Clin. Nutr.* 68(S) (1998).
2. Harry J.B. and Steiner R.F. A Soybean Proteinase Inhibitor Thermodynamic and Kinetic Parameters of Association with Enzymes *Eur. J. Biochem.* 16, 174-179 (1970).

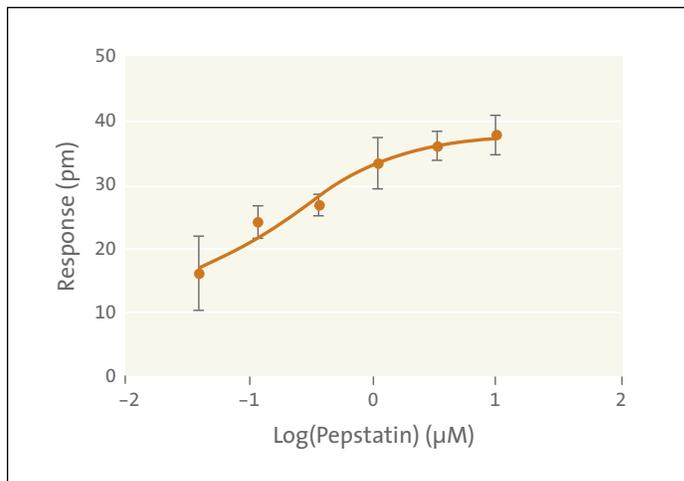


Figure 6. Pepsin/pepstatin dose response binding curve of cationized protein with ethylenediamine (ED) as described in materials and methods.

3. Schlamowitz M and Peterson L.U. Studies on the optimum pH for the action of pepsin on “native” and denatured bovine serum Albumin and bovine hemoglobin. *J. Biol. Chem.* 234, 3137-3145 (1959).
4. Lin X., Wong R.N.S., and Tang J. Synthesis, Purification and Active Site Mutagenesis of Recombinant Porcine Pepsinogen. *J. Biol. Chem.* 264, 4482-4489 (1989).

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