Investigation of enhancement in thermal stability of trypsin in modified Mg/Al layered double hydroxides

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Plot of percent retention of activity vs. temperature

Highlights

- Trypsin was immobilized in Mg/Al layered double hydroxides (LDHs) using three methods.
- Trypsin entrapped between LDHs modified with Sodium Dodecyl Sulphate showed the highest stability against autolysis.
- Delicate modifications of LDH structure provides a promising method of long-term storing of trypsin at room temperature.

RESEARCH ARTICLE

Investigation of enhancement in thermal stability of trypsin in modified Mg/Al layered double hydroxides

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Abstract: Mg/Al Layered Double Hydroxides (LDHs) were functionalized to immobilize the serine protease, trypsin. Three methods were implemented for immobilization: physical adsorption, entrapment and covalent cross-linking. Trypsin was immobilized in Mg/Al-NO₃⁻ LDH via simple adsorption. The same LDH host was modified with Sodium Dodecyl Sulphate (SDS) which assembles inside the double layer, for the enzyme to be entrapped. For covalent cross linking, LDH host was modified with vertical pillars of glutamate ions which were cross-linked to horizontally aligned dicarbonyl linkers. This cross linkage firmly holds the enzyme via Schiff's base linkages using the free amino groups of the enzyme. Thermal stability and storage stability of the immobilized enzyme was studied in comparison with the free enzyme using trypsin activity assay experiments. The enzyme showed remarkable stability against autolysis even at higher temperatures showing the potential of modified LDHs to store trypsin at room temperature.

Keywords: Trypsin; autolysis; immobilization; LDH; thermal stability.

INTRODUCTION

Layered Double Hydroxides (LDHs) are well known in immobilizing biologically active materials such as porphyrins (Park *et al.*, 1989; Bonnet *et al.*, 1996; Robbins and Dutta, 1996), nucleoside phosphates (Choy *et al.*, 2000; Choy *et al.*, 2001; Minagawa *et al.*, 2019), drugs (Fardella *et al.*, 1997^a; Fardella *et al.*, 1997^b; Fardella *et al.*, 1998; Fogg *et al.*, 1998; Khan *et al.*, 2001; Choi *et al.*, 2018), vitamins (Hwang *et al.*, 2001), amino acids (Whilton *et al.*, 1997; Aisawa *et al.*, 2000; Aisawa *et al.*, 2001) and fatty acids (Meyn *et al.*, 1990; Borja and Dutta, 1992). It is believed that the host lattice may protect these relatively delicate, but comparatively large biomolecules from degradation and also aid their transport to specific targets within the body (Mansouri *et al.*, 2009).

Once an enzyme is purified outside its native biological environment, it is liable to denature due to deviations of temperature, pH value, radiation and various other factors. Proteases possess an added affinity to lose its maximum catalytic capacity via destruction of its native protein structure by their own action of proteolysis called

autolysis (Koutsopoulos *et al.*, 2007). In biology, autolysis refers to the destruction of a cell through the action of its own enzymes. It may also refer to the digestion of an enzyme by another molecule of the same enzyme. Trypsin being a protease should be stored at low temperatures (between -20 °C and -80 °C) to prevent autolysis. Autolysis may also be prevented by storage of trypsin at pH=3 or by modifying trypsin via reductive methylation. When the pH is adjusted back to pH=8, the activity returns back to normal (Koutsopoulos *et al.*, 2007).

Immobilization serves as an alternative way to prevent autolysis (Homaei *et al.*, 2013). An immobilized enzyme is an enzyme which is attached to an inert, insoluble material. Trypsin has previously been immobilized on variety of supports such as polymeric resins, natural polymeric derivatives or organic gels and fibers (Sheldon, 2007). Most of the host materials are of limited capacity for reuse, creating problems of disposing organic materials such as polysaccharides, polyacrylic and polyvinyl materials (Zucca and Sanjust, 2014). On the other hand, inorganic carriers (silica, silicates, borosilicates, aluminosilicates, alumina, titania and etc) have the advantage of being reusable and are generally resistant to microbial attack and not affected by changes in pH or swollen by organic solvents (Jaros and Rohm, 2011; Zucca and Sanjust, 2014).

Trypsin (EC 3.4.21.4) is one of the major pancreatic proteases belonging to the diverse serine protease family. It is synthesized in the form of inactive proenzyme trypsinogen (Chen *et al.*, 2009; Saluja *et al.*, 2019). Trypsin plays a central role in pancreatic exocrine physiology by triggering the activation of all other pancreatic digestive zymogens, including its own inactive precursor (Halfon *et al.*, 2004). Furthermore, serine proteases are the most widely studied group among the common proteases and more precisely trypsin can be considered as a model enzyme since it has been characterized and employed extensively in research (Oliveira *et al.*, 2005; Heissel *et al.*, 2019). Trypsin is the usual enzyme of choice for passaging cells in culture and in development of cell and tissue culture protocols (Zhou *et al.*, 2020; Banumathi *et al.*, 2009). Since it reliably cleaves peptide chains with defined specificity, trypsin is extensively used in protein sequencing protocols and other

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proteomic applications (Schuchert-Shi and Hauser, 2009). In the field of medicine, the role of trypsin in pancreatic diseases, including cystic fibrosis and chronic pancreatitis has been the subject of current research, and trypsin has been used to model the decomposition of articular cartilage in osteoarthritis (Chen *et al.*, 2009; Wang *et al.*, 2008). Trypsin contains a catalytic triad consisting of histidine-57, aspartate-102, and serine-195. These three residues form a charge relay which serves to make the active site serine, nucleophilic. This is achieved by modifying the electrostatic environment of the serine (Polgár, 2005).

There are three different ways in which one can immobilize an enzyme, and their effectiveness differs depending on the strength of immobilization power and their capacity to transport the substrate to the interior and the soluble products to the exterior. The simplest and cheapest by far is the physical adsorption method. Enzyme is attached to the outside of an inert material. As adsorption is not a chemical process, the active site of the immobilized enzyme may be blocked by the matrix or bead, greatly reducing the activity of the enzyme (Beck *et al.*, 1992). Entrapment is the second method and the enzyme is trapped in insoluble beads or microspheres, such as calcium alginate beads. However, these insoluble substances can hinder the arrival of the substrate, and the exit of products (Hakala *et al.*, 2013). When the enzyme is covalently bonded to a matrix through a chemical reaction, it can be referred to as covalent cross-linking. This method is by far the most effective method. As the chemical reaction ensures that the binding site does not cover the enzyme's active site, the activity of the enzyme is only affected by immobility (Bai *et al.*, 2012; Schuckel *et al.*, 2011).

In this study, an attempt was made to immobilize trypsin in Mg/Al layered double hydroxides using the three methods mentioned above. The unmodified lattice was used to intercalate trypsin via physical adsorption method and for entrapment and covalent cross linking the lattice was modified. Herein, we propose a method to store trypsin at room temperature despite environmental fluctuations and its own action of autolysis.

MATERIALS AND METHODS

Materials

Bovine Trypsin, Sodium Dodecyl Sulphate (SDS), 1, 3-butandiol, Trichloroacetic acid (TCA) and L-Glutamic acid was purchased from Sigma Aldrich. All other chemicals used were of analytical grade.

Preparation of Mg/Al- NO_3^- LDH

Mg/Al- NO_3^- LDH basic host lattice was synthesized using $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ and $\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ with the molar ratio of Mg:Al = 2:1. First, a clean dry 500 mL three necked flask was equipped with a pH meter probe and two separatory funnels to build up the apparatus. The system was cleaned and flushed with a stream of N_2 (g) for a sufficient period of time to ensure the environment exposed to the system was free from CO_2 (g). The 0.2 and 0.1 mol dm^{-3} cationic

solutions (100.0 mL) were allowed to mix with 1.00 mol dm^{-3} NaOH maintaining the pH at 10.0. The slurry was heated at 65 °C and aged for 18 h while stirring continuously. The semi-solid was cooled, filtered and washed with distilled water for several times and dried at 70 °C for 24 h using the vacuum oven.

Preparation of Mg/Al-SDS-LDH

Mg/Al-SDS-LDH lattice was prepared by co-precipitation method according to the method described in above using the same cationic solutions and the solution of alkali. The anionic surfactant SDS was incorporated into the cationic lattice with simultaneous mixing of the above solutions along with drop wise addition of 0.1 mol dm^{-3} SDS solution (100.0 mL) maintaining the pH at 10. The slurry was aged for 18 h and dried at 70 °C for 24 h under vacuum.

Preparation of Mg/Al-Glutamate pillared LDH

Preparation of Mg/Al- CO_3^{2-} LDH

Mg/Al- CO_3^{2-} LDH was prepared as the initial host lattice for Mg/Al-Glutamate pillared LDH using the co-precipitation method described earlier incorporating CO_3^{2-} ions using a 1.0 mol dm^{-3} NaOH/ Na_2CO_3 solution (100.0 mL) as alkali.

Preparation of glutamate pillared LDH

4.00 g of the Mg/Al- CO_3^{2-} LDH prepared above was dissolved in 100.0 mL of distilled water and the suspension was stirred until it was dissolved with effervescence to give a clear solution. This clear solution was allowed to be mixed with a 0.5 mol dm^{-3} NaOH solution in a round bottomed flask. The pH of the solution was maintained above 9.0 by regulating the addition of NaOH in a drop wise manner. The resulting slurry was refluxed for 6 h by attaching a condenser and the solid formed was recovered by filtration. Collected solid was rinsed several times with distilled water and was dried at 75 °C for 24 h under vacuum.

Immobilization of trypsin in LDHs

Immobilization of trypsin in Mg/Al- NO_3^- and Mg/Al-SDS LDHs

Dried, finely powdered LDHs (5.00 g) were transferred to 5.00 mL of freshly prepared phosphate buffer (pH=7.6) and distilled water (5.00 mL) separately and the suspensions were stirred magnetically for 10 min at 50 rpm. Then, 5.00 mL portions of trypsin solution (0.1250 g of trypsin in 25.00 mL of 0.001 mol dm^{-3} HCl) were added to the mixtures while stirring. Stirring was continued for 2 h at 100 rpm at room temperature. The immobilized enzymes were recovered by centrifuging the suspensions at 6000 rpm for 10 minutes. The supernatants were kept to carry out the trypsin activity assay. The solid recovered was washed thoroughly for several times with distilled water and was dried at room temperature and was stored inside a desiccator.

Immobilization of trypsin in glutamate pillared LDH

An aqueous solution of 3-oxobutanal (10.00 mL, 5 % v/v) was added to a suspension of glutamate-pillared LDH (5.00 g in 50.00 mL of distilled water) and was stirred magnetically at 55 °C for 1 h. The resulting solid was filtered and washed several times with distilled water. Then, the immobilization procedure described above was followed with the solid recovered.

Structure optimization

Geometry optimization via semi empirical method was carried out with the Gaussian software. Geometry optimization began at its molecular structure specified as its input and stepped along the potential energy surface. It computed the energy and the gradient at that point and determined how far and in which direction to make the next step. The gradient indicated the direction along the surface in which the energy decreases most rapidly from the current point as well as the steepness of the slope.

Trypsin activity assay

The activity of trypsin was estimated by determining the amount of casein cleaved by trypsin. 280.0 µL of freshly prepared phosphate buffer (pH=7.6) was transferred to an autoclaved micro-centrifuge tube and 20.0 µL of the trypsin solution was added and mixed. The buffer- trypsin mixture and 1% casein solution were pre-incubated separately at 37 °C for 10 min. Then 400.0 µL of 1% pre incubated casein solution was added to the trypsin solution and was further incubated for an hour at 37 °C. Then, the reaction was quenched using 800.0 µL of 5% trichloroacetic acid (TCA). The mixture was centrifuged for 10 min at a rate of 1×10^4 rpm and the absorbance of the supernatant was measured at 285 nm using the UV visible spectrophotometer using the phosphate buffer as the reference. The activity calculated is equivalent to number of enzyme units (U) which cleaves 1 µmol of substrate (casein) per minute at pH = 7.6 at 37 °C.

The specific activity calculated was expressed as number

$$\text{Activity(U)} = \frac{\Delta O_{285}}{t_{\text{incubation}} \times n_{\text{substrate}}}$$

ΔO_{285} = (absorbance of the sample at λ_{285})-(absorbance of the blank at λ_{285})

$t_{\text{incubation}}$ = incubation time/min

$n_{\text{substrate}}$ = amount of substrate/µmol

Percent immobilization yield and residual activity

Percent immobilization/inclusion was calculated according

$$\text{Percentage immobilization yield} = \frac{(U_{\text{initial}} - U_{\text{final}})}{U_{\text{initial}}} \times 100\%$$

U_{initial} = Initial amount of enzyme units used

U_{final} = Enzyme units remaining in the supernatant after immobilization

Trypsin assay was performed using 0.5000 g of the dried solid immobilized enzyme samples instead of 20.0 µL of free trypsin in solution. Then the residual activity and the residual specific activity of each sample were calculated in triplicates.

Thermal stability

Immobilized trypsin samples and free trypsin in solution in micro centrifuge tubes were incubated for 1 h at temperatures: 37.0, 50.0, 60.0, and 70.0 °C. The trypsin activity assay was performed using 0.5000 g of solid immobilized trypsin and 20.0 µL of free trypsin in solution. The residual activity and residual specific activity were calculated in triplicate in each case and the average value was calculated.

Long term-thermal stability and storage stability

The procedure described in thermal stability was followed by extending the storage time to 32 days. The incubation was carried out at 50.0 °C and free trypsin was stored at 50.0 °C both in solid and solution forms. Samples for the assay were taken out on day 1, 2, 3, 7, 9, 21 and 32 and the activity and specific activity of each sample were calculated in triplicate and the average value and the standard deviation were calculated. The free solid trypsin stored at 50.0 °C was taken out and a solution of similar concentration to the trypsin stock solution was prepared prior to the trypsin activity assay.

Characterization of solid products

The PXRD patterns and FT-IR spectra of solid products were used to monitor the formation of intermediates at each step. FT-IR spectra were recorded on Nicolet Nexus 6700 spectrometer (Thermo Nicolet Corporation, USA) under scan range 400-4000 cm^{-1} using pellets (1/10 wt.%). As the reference, Mg/Al- unmodified host lattice was used. All X-ray powder diffraction patterns (PXRD) were collected with an X-Ray diffractometer (Siemens D5000, Germany) equipped with a Cu anode and Ni K β filter ($K\alpha$, $\lambda = 0.154$ nm), operating at 40 KV and 40 mA over 2θ range from 2° to 40°)

RESULTS AND DISCUSSION

The major focus of this work was to determine the effects of modifications inside the gallery spaces of Mg/Al LDHs to enhance the immobilizing power.

In all PXRD patterns recorded for the solid products, the diffraction planes were indexed on the basis of a hexagonal unit cell type with R-3m rhombohedral symmetry. Three major diffraction peaks were clearly visible in the PXRD patterns obtained for the solid materials and they were characteristic to the basic structure of LDHs. Broad peaks found in the region below 10° indicate a possible heterogeneity of the interlayer spacing in both parental and intercalated include LDHs. These peaks can arise due to the expansions of planes of (003) in different magnitudes in the samples analyzed (Zheng *et al*, 2017). Further, all PXRD patterns depict a significant level of noises indicating a low degree of crystallinity in the samples synthesized.

Figure 1.a: Powder XRD patterns of (1) Mg/Al NO₃· LDH (2) Trypsin adsorbed on Mg/ AlNO₃· LDH

In Mg/Al-NO₃· LDH, the characteristic peak present in high intensity at 2θ value of 11.36° ($d=7.84 \text{ \AA}$) for (003) plane along with other two significant peaks at 2θ values of 19.46° and 34.14° for (006) and (009) respectively confirms the occurrence of interlayer NO₃⁻ anions (Figure 1.a.1). Literature values indicate that the thickness of the brucite-like cationic layers of the LDH host is $4.8 \text{ \AA}$, thus the gallery height of $3.0 \text{ \AA}$ accommodates the anion NO₃⁻ which possess a comparable size (Chakraborty *et al.*, 2017). FT-IR spectrum of Mg/Al-NO₃· LDH (Figure 1.b.1) shows a broad absorption peak in the region of $2800\text{--}3600 \text{ cm}^{-1}$. It denotes the presence of hydroxyl groups and inter-layer water molecules which are in a continuous exchange process. The deformation vibrations of the LDH basal layer are given by the peak at 1615 cm^{-1} . In the low frequency region, the absorption peaks at 425 cm^{-1} , 620 cm^{-1} and 830 cm^{-1} give the lattice vibration modes which attribute to M-O and O-M-O. The very strong and sharp peak at 1385 cm^{-1} is characteristic to stretching of nitrate group. Physical adsorption method was implied to immobilize the enzyme in this unmodified host lattice. In the PXRD pattern of trypsin adsorbed LDH, the lattice expands to $40.6 \text{ \AA}$ in maximum as a result of first order intercalation and accordingly the respective gallery height is $35.8 \text{ \AA}$ (Figure 1.a.2). Trypsin possesses a size in the range of $\sim 40 \text{ \AA}$ the gallery expansion is comparable (Islam *et al.*, 2013). In order to enhance further clarity, authors used PyMol software to estimate the dimensions of crystal lattice of trypsin. Accordingly, trypsin has the dimensions of $48 \times 43 \times 34 \text{ \AA}$. Gallery height is such that the molecule can be incorporated into the double layer without grafting. However, the appearance of another strong peak at 2θ value of 6.81° for (003) indicates the second stage intercalation of Trypsin in some LDHs in the heterogeneous mixture (Zheng *et al.*, 2017). Figure 1.b.2 gives FT-IR evidence for the occurrence of trypsin in gallery spaces. The reduced peak strength at 1385 cm^{-1} gives an indication that some nitrate ions has been replaced. Trypsin being an enzyme with a tertiary protein structure should show characteristic peptide bond vibrations and symmetric and asymmetric stretches of alkyl -CH groups. The new peak at 1635 cm^{-1} gives evidence for the peptide -CONH bonds in the interlayer region. The weak shoulder

Figure 1.b: FTIR spectra of (1) Mg/AlNO₃· LDH (2) Trypsin adsorbed on Mg/ AlNO₃· LDH

peak at 2950 cm^{-1} must be due to the alkyl symmetric -CH stretch of the enzyme.

During the first modification of the LDH host lattice, an entrapping agent was introduced first, in order to modify the gallery spaces. SDS which was used as the entrapping agent possesses a hydrophobic tail and a hydrophilic head. After inserting the SDS chains into the LDH host lattice, the d-space expanded to $36.2 \text{ \AA}$ and the gallery height is therefore $31.4 \text{ \AA}$ (Figure 2.a.2). According to the literature, the average length of a SDS chain is $15 \text{ \AA}$ (Cabane *et al.*, 1985). Thus, we inferred that SDS chains align in a double layer (Islam *et al.*, 2013), within the gallery spaces. This double layer arrangement is solely due to the amphiphilic nature of the SDS chains. SDS hydrophilic head is negatively charged and the head regions can align vertically in the positively charged layers of the LDH host lattice and the inherent repelling forces will align the hydrophobic tails towards the middle of the gallery space. Thus, the hydrophobic tails of the bilayer face themselves repelling the cationic layers.

In the FT-IR spectra (Figure 2.b.2), the new peak immersed at 2916 cm^{-1} gives an indication of newly intercalated species. This peak is relevant to the symmetric alkyl stretches of SDS chain and the peak at 1460 cm^{-1} is related to the anti-symmetric alkyl stretches. Further the peak at 1030 cm^{-1} indicates the sulfate stretch giving additional evidence to the intercalation of SDS within the gallery space. The SDS bilayer provides an effective entrapping network to hold the enzyme in place.

After immobilizing the enzyme, the gallery height has further extended to $42.0 \text{ \AA}$ showing the successful insertion of trypsin via entrapment (Figure 2.a.3). On the other hand, the disappearance of the prominent peak at 2θ , 11.36° of the parental PXRD pattern (NO₃⁻-LDH) in the modified forms proved the formation of complete first stage intercalation.

In the FT-IR spectrum (figure2,b), it is clearly visible that a new peak has emerged at 1635 cm^{-1} and this accounts for the bond vibrations in -CONH peptide bonds in the primary peptide chains of the enzyme.

There is an enhancement in the alkyl anti-symmetric

Figure 2.a : Powder XRD patterns of (1) Mg/ Al NO_3 ·LDH (2) Mg/ Al SDS LDH (3) Trypsin entrapped within Mg/ Al SDS LDH

Figure 3.a: Powder XRD patterns of (1) Mg/ Al CO_3^{2-} LDH (2) Glutamate pillared LDH (3) Covalently linked trypsin within GP LDH

stretches in the region centering 1438 cm^{-1} . This gives evidence for the entrapment of more organic material in the gallery space. The peaks relevant to SDS remain intact ensuring that the SDS chains are adhering within the gallery space even after entrapment of trypsin.

The second modification was performed in order to immobilize trypsin within the LDH host using a firm covalent link via a formation of a Schiff's base linkage. This Schiff's base link was allowed to form between the free amino group of the enzyme and the free carbonyl group of the dicarbonyl cross linker which is bound to the free amino groups of the glutamate pillars (GP) vertically aligned in the gallery space.

Mg/Al- CO_3^{2-} LDH was synthesized as the initial host. Unlike nitrate ions, carbonate ions have a higher affinity for intercalation. If nitrate is used instead, there will be a higher replacement of nitrate ions with glutamate anions. Therefore, Mg/Al- CO_3^{2-} LDH was chosen as an ideal starting material to modify the interlayer region with loosely packed glutamate pillars.

In the Mg/ Al - CO_3^{2-} LDH, three main peaks are at 20°

Figure 2.b: FTIR spectra of (1) Mg/ Al NO_3 ·LDH (2) Mg/ Al SDS LDH (3) Trypsin entrapped within Mg/ Al SDS LDH

Figure 3.b: (1) Mg/ Al CO_3^{2-} LDH (2) Glutamate pillared LDH (3) Covalently linked trypsin within GPLDH

11.67° , 23.17° and 34.67° corresponding to the typical reflection planes of (003), (006) and (009), respectively (Figure 3.a.1). The major peak $d_{003}=7.6\text{ \AA}$ gives the basal spacing value of a characteristic carbonate LDH (Figure 3.a.1) which corresponds to a gallery height of $2.8\text{ \AA}$ compatible with the size of carbonate ions (Ren *et al.*, 2002). After intercalating glutamate ions as the pillaring agent, the d-spacing has expanded to $16.8\text{ \AA}$ denoting a gallery height of $12.0\text{ \AA}$ (Figure 3.a.2) and shifted slightly three major peaks found in the parental LDH to lower 2θ values indicating the layer expansion. The average of the glutamate anion is approximately $10\text{ \AA}$ which is comparable with the expansion of the gallery height (Wu *et al.*, 2020).

Once the cross-linking agent synthesized was introduced to the lattice, the enzyme was allowed to bind to the cross linker via covalent Schiff's base linkage. After sequential intercalation of these species, the observed d-spacing was $42\text{ \AA}$ with a gallery height of $37\text{ \AA}$ (Figure 3.a.3). This value is comparable with the size of trypsin and gives evidence for the successful immobilization of the enzyme within the gallery space.

The sharp intense peak at 1377 cm^{-1} can be assigned

Figure 4: (a) Geometry optimized structure for the intermediate cross linked stage of GP-LDH (b) covalently linked enzyme to the dicarbonyl cross linker

Table 1: Summary of percent immobilization yield

Sample	Average activity of the supernatant solution / U		Percent Immobilization
	Before immobilization	After immobilization	Yield/%
Mg/Al- NO_3^- LDH	1545.20	757.83	50.96
Mg/Al-SDS LDH	1545.20	268.92	82.60
GP LDH	1545.20	346.96	77.55

Table 2: Average residual and specific activities of immobilized enzyme samples of 0.0500 g of dry weight

Sample	Average residual activity/U	Average residual specific activity/U g
Mg/Al- NO_3^- LDH	4.12	82.50
Mg/Al-SDS LDH	31.28	625.66
GP LDH	5.75	115.02

to the carbonate bond vibrations in the interlayer space (Figure 3.b.1). The broad absorption peak centered at 3471 cm^{-1} denotes the vibrations of hydroxyl groups which are in a continuous exchange process with water molecules. After pillaring the gallery space with glutamate ions immergence of new peaks can be observed at 1594 cm^{-1} and 1405 cm^{-1} (Figure 3.b.2). They are assigned to the RCOO^- symmetric and anti-symmetric stretches respectively. The shoulder peak at 3040 cm^{-1} indicates the symmetric stretch of alkyl $-\text{CH}$ groups of the glutamate dianion.

The immergence of the peak at 1639 cm^{-1} which can be assigned to the $-\text{C}=\text{N}$ Schiff's base bond vibration (Figure 3.b.3). This is the major evidence to indicate that the free amino groups of trypsin may have successfully bound to the free carbonyl end of the dicarbonyl linker. This peak was not observed in previous spectra thus it is clear that in the third step after introducing the dicarbonyl linker, it has served as a supportive agent to firmly hold the enzyme via covalent bond formation.

Figure 4 indicates the molecular models of the glutamate pillar in its geometry optimized structure and how the enzyme links to the dicarbonyl linker.

Percent immobilization yield

The percent immobilization yield was calculated by evaluating the number of active enzyme units remaining in the supernatant after immobilizing trypsin inside the host lattices. The remaining active enzyme units were subtracted from the initial amount of enzyme units used for the immobilization process. According to the Table 1, it clearly indicates that the modified LDH host lattices afford a higher immobilization yield compared to the unmodified host lattice. Mg/Al-SDS-LDH modified for entrapment of trypsin affords the highest immobilization yield.

Residual specific activity of immobilized trypsin

According to the Table 2, modified LDH host lattices afford a higher retention of activity compared to the unmodified host lattices. Among the two modified host lattices, the SDS entrapped trypsin showed the highest retention of activity. That is due to the retention of active pocket conformation by firm entrapment of SDS self-assembly.

Table 3: Percent retention of activity in immobilized trypsin and trypsin in free solution

Sample	Percent retention of activity/%			
	Temperature/ °C			
	37.0	50.0	60.0	70.0
Free trypsin in solution	100.0	66.0	45.8	19.2
Mg/Al- NO ₃ LDH	100.0	84.2	76.8	75.0
Mg/Al-SDS LDH	100.0	97.1	96.1	93.9
GP LDH	100.0	95.0	90.4	86.4

Figure 5: Plot of percent retention of activity vs. temperature.

Figure 6: Plot of percent retention of activity at 50.0 °C vs. storage time.

Figure 7: Schematic diagram illustrating immobilization of enzyme via entrapment of SDS self-assembly.

Thermal stability and storage stability of trypsin in free and immobilized forms

According to Figure 5 and Table 3, there was a drastic reduction in activity when the temperature was increased starting from the room temperature, for the free form of trypsin. However there was a significant retention in activity in immobilized trypsin, even at higher temperatures like 60.0 °C and 70.0 °C.

The modified LDH lattices showed a better stability for entrapped trypsin. According to the experimental data obtained after each trial of trypsin activity assay, it is clearly visible that there is a significant retention in activity once trypsin is immobilized within a host material other than it is being disclosed to fluctuations in environmental factors such as temperature. Further, there is a clear evidence to show that the modified LDH host lattices are far more effective than the unmodified host lattices (Figure 6). Being a proteolytic enzyme, trypsin is usually subjected to autolysis (Bunkenborg *et al.*, 2013). When entrapped within modified lattices, it reduces the intermolecular collisions and hence it will impart a better stability in the entrapped trypsin than the free trypsin.

Further, enhancements in the covalent cross-linking method may protect the delicate configurations within the active pocket since it can firmly hold the enzyme via covalent bonding throughout the reaction. (Nguyena and Kima, 2017). The pillaring agent should be more effective if the length of the pillar is comparable with the enzyme size. The pillar used in this attempt is shorter than the enzyme size. Therefore there is a natural tendency for the ionic interactions to be weakened after attachment of the bulky trypsin molecule.

On the other hand, SDS self-assembly effectively protects the trypsin active pocket conformation by its hydrophobic network of chains aligned in a double layer (Figure 7). This loosely packed network facilitates transfer of substrate and subsequently the products without blocking the free entry of these species.

CONCLUSION

Application of LDHs in immobilization of trypsin provides

a reliable way to aid in retention of activity minimizing autolysis to a significant extent. This simple and inexpensive method retained the enzyme performance even at temperatures like 70 °C. SDS entrapped trypsin gave the highest retention of activity (84.66%) while covalent cross-linking can further be improved. Delicate modifications of LDH structure may provide a promising method of storing enzymes at room temperature.

Future studies may be extended to investigate modifications of the cross-linking agent. Determination of the capacity of immobilized trypsin to function in organic solvents is another conceivable area to extend the studies. The reliability of these host materials for immobilization of other proteases and non-proteases can be investigated for the vast applicability of clinical purposes.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

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