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Covalent Immobilization of Trypsin on a Novel Aldehyde-Terminated PAMAM Dendrimer

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Dendrimers are a novel class of nonlinear polymers and due to their extensive applications in different fields, called versatile polymers. Polyamidoamine (PAMAM) dendrimers are one of the most important dendrimers that have many applications in nanobiotechnology and industry. Generally aldehyde terminated dendrimers are prepared by activation of amine terminated dendrimers by glutaraldehyde which has two problems, toxicity and possibility of crosslink formation. In this study, novel aldehyde-terminated PAMAM dendrimer was prepared and used for covalent immobilization of trypsin by the aim of finding a special reagent which can prevent crosslinking and deactivation of the enzyme. For this purpose aminoacetaldehydedimethylacetal (AADA) was used as spacer group between aldehyde-terminated PAMAM and trypsin.The findings of this study showed that immobilization of trypsin not only resulted higher optimal temperature, but also increased the thermal stability of the immobilized enzyme in comparison to the free enzyme.

Key Words : Immobilization, Trypsin, Synthesis, PAMAM dendrimer, Aldehyde terminated dendrimers

Introduction

Dendrimers are a group of organic polymers which have regular and defined structure. These unique macromolecules have low polydispersity, and commonly created with nanometer dimensions (1 to 10 nm). The management of size, shape, and surface functionality makes dendrimers one of the "smartest" biomaterials for nanotechnological applications. Also, the structure of these macromolecules has a great effect on their physical and chemical properties.[1] Many of the applications include the covalent coupling of functional groups of dendrimers with other compounds and form bioconjugate. These bioconjugates can act as detection agents, affinity ligands, targeting molecules, radio-ligands, imaging agents and *etc*. The methods for dendrimer conjugation are similar to the procedures used with other nanoparticles. The essential elements in designing a dendrimer conjugate are the functional groups that are present on the dendrimer and the functional groups on the nanoparticles to be coupled. Biologically active macromolecules which loaded on dendrimers have a wide range of applications in nanomedicine.[2-4]

Dendrimers are composed of a core, branching units and surface functional groups. Their size, shape and reactivity are assigned by generation, chemical structure of the core, size of branching units and surface functionalities. The number of terminal groups increases with each series or generation (G) of the synthesis.[5-7]

Polyamidoamine (PAMAM) dendrimers are one of the most important dendrimers which have many applications in biomedicine and nanomedicine.[8-12] These applications include drug delivery, gene delivery, enzyme and protein immobilization, magnetic resonance imaging (MRI), dendrimer-protein conjugates, dendrimer-peptide conjugates, *etc*.[5,6,8,11,13-15] These unique nonlinear polymers have many functional end groups that can be converted to other groups by functional group inversion (FGI) method. In most cases of the applications of dendrimers, molecules are covalently linked to dendrimer surface or coupled with the functional components in the branched structure of dendrimer.[13-17] The essential element in designing a dendrimer-protein conjugate is the functional end groups present on the surface of dendrimer, and the functional groups on the proteins to be coupled. PAMAM dendrimers have different functional groups including amine, carboxylate, hydroxyl, ester and other possible moieties. Existing method for preparation of aldehyde terminated dendrimers is activation of amine terminated dendrimers by glutaraldehyde which has two complicated problems, first is toxicity of glutaraldehyde and second is possibility of crosslink because of existing two aldehyde functional groups on its both ends. So, the aim of this research is finding a special reagent which is protected on one end and has an amine group on the other end. This special reagent is (AADA) that reacted with ester terminated PAMAM dendrimer (half generation) (Fig. 1) and then hydrolyzed with trifluoroacetic acid (TFA). The final product will be PAMAM dendrimer with aldehyde terminal groups (polyamidoaldehyde dendrimer or PAMAL dendrimer). This dendrimer has ability to react with biological macromolecules such as DNA, proteins, peptides, antibodies, drugs

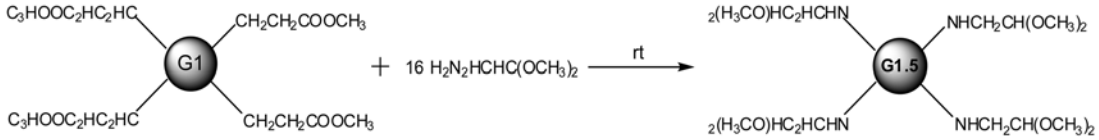

Figure 1. Synthesis pathway of (G1.5) pamam acetal terminated dendrimer: dropwise addition of AADA vigorously stirred at 0 °C to 25 °C under nitrogen in methanol, 96 h.

and *etc*.[4,18,19]

Nowadays, using immobilization of enzymes by various carriers like polymers has many applications in biological reactors and enzymatic sensors. Therefore, enzymatic immobilization methods are as an interesting field for researchers as a key stage in enzyme modification.[20-22]

The use of free enzymes in biological applications is restricted due to its high price, and impossibility of recovering and separating them from the reaction medium after the enzymatic process. One possible solution for these problems is enzyme immobilization in order to separate catalyst from the reaction medium. Immobilized enzymes have excellent properties such as resistance to temperature and denaturants, and longer stability compared to free enzymes.[21,23]

In this research polyamidoamine dendrimers from G0.5 (with 4 ester terminal functional groups) to G2 (with 16 amine terminal functional groups) were synthesized. Then, G1.5 with (16 ester terminal functional groups) reacted with aminoacetaldehyde dimethyl acetal to convert ester terminal functional groups to acetal terminal functional groups. Finally, acetal terminal groups were hydrolyzed by TFA to aldehyde terminal groups. Synthesized polyamidoaldehyde (PAMAL) has many applications in enzyme immobilization, drug delivery, protein conjugates, *etc*.[23]

In this study the potential of novel dendrimer nanocarrier that can attach to proteins and forms protein-polymer bioconjugates has also been studied. Trypsin can be considered as a model protein. With dendrimer nanoparticle technology, nanocarriers aim fto imprve protein stability and its therapeutic efficacy.

Experimental

Methanol, ethylenediamine (EDA), methylacrylate, hydrochloric acid, trifluoroacetic acid (TFA), aminoacetaldehyde dimethyl acetal, dimethyle sulfoxide (DMSO), DMSO-d_6 and deuterated chloroform ($CDCl_3$), fetal bovine serum (FBS) were purchased from Merck (Darmstadt, Germany) and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), MCF-7 cell, RPMI-1640, penicillin and streptomycin were purchased from Sigma, MCF-7 cell line (kindly donate by the Pharmaceutical Nanotechnology Research Center Tabriz University of Medical sciences, Tabriz, Iran) and trypsin was prepared from Gibco® - Invitrogen, Canada. All chemicals were analytical grade and used without further purification except for ethylenediamine which was distilled before using.

^{1}H NMR and ^{13}C NMR spectra were recorded with Bruker 300 and 400 MHz, FTIR spectra, scanned with Shimadzu 4300 FTIR spectrophotometer from 4000-400 cm^{-1}. Solvent excess EDA removed by vacuum rotary evaporator (Heidolph Laborota 4003, Schwabach, Germany) and products were purified by vacuum dry oven (DK-VDO001, Korea). Enzyme activity was measured by UV-visible spectrophotometer (UV-2550 Shimadzu, Japan.

Synthesis of (G-0.5) PAMAM Dendrimer with Ethylenediamine Core. The solution of freshly distilled EDA (9.9 g, 0.165 M) in methanol (40 mL) under nitrogen was added dropwise to a stirred solution of methylacrylate (74 mL, 0.816 M) in methanol (40 mL over a period of 3 hrs. The resulted mixture was stirred for 60 min at 0 °C and then allowed to reach room temperature and stirred for a further 24 hrs. The solvent was removed under reduced pressure at 40 °C with a rotary evaporator and the colorless oily product was dried under vacuum (2 mm Hg, 50 °C) for 10 hrs to give the nal product (58 g, yield; 86%).[23]

($^aCH_2{}^aCH_2$)[N($^bCH_2{}^cCH_2{}^dCO_2{}^eCH_3$)$_2$]$_2$: FTIR ν ($cm^{-1}$): 1745-1730; (CO); ^{1}H NMR ($CDCl_3$) δ_H 3.62 (12H, s, CH_3), (2.72, 8H, t, N-CH_2), 2.45 (4H, s, CH_2-N, CH_2-CH_2), 2.40 (8H, t, CH_2-CO); ^{13}C NMR ($CDCl_3$) δ_C (171.88 (d), 51.14 (e), 50.45 (b), 48.65 (a), 31.52 (c).

Synthesis of (G0) PAMAM Dendrimer. G-0.5 PAMAM dendrimer (15 g, 0.024 mol) was dissolved in methanol (30 mL) and was added gradually to a strongly stirred solution of EDA (128 mL, 115.2 g, 1.91 mol) in methanol (150 mL) at 0 °C. After complete addition of the dendrimer solution, the mixture was stirred for 5 days at room temperature. The methanol was removed under reduced pressure below 40 °C. The excess of EDA was removed by azeotropic distillation with toluene and methanol (95:5). Toluene was removed by azeotropic distillation using methanol. lastly, removal of the remaining methanol under vacuum (2 mm Hg, 40 °C, 48 hrs) gave the four amine terminal group PAMAM, named G0 as a colorless highly viscous and adhesive product (yield; 85%).[23]

($^aCH_2{}^aCH_2$)[N($^bCH_2{}^cCH_2{}^dCO^eNH^fCH_2{}^gCH_2{}^hNH_2$)$_2$]$_2$: IR (ν/$cm^{-1}$): 1645 (amide C=O), 3300 (NH_2), 3100 (NH); ^{1}H NMR ($CDCl_3$) δ_H 7.67 (12H, s, N-H), 3.23 (8H, bq, N-CH_2), 2.68 (8H, t, N-CH_2), 2.55 (8H, t, CH_2-CO), 2.31 (4H, s, CH_2-N), 2.24 (8H, t, NH_2), 1.48 (8H, p, CH_2-NH_2); ^{13}C NMR ($CDCl_3$) δ_C 171.97 (d), 171.41 (j), 51.77 (k), 50.53 (f), 49.01 (b) 48.14 (g).

Synthesis of (G1.5) PAMAM Dendrimer with Ester Terminated Groups. The solution of (G1); (9.75 g, 0.0068 mol) in methanol (30 mL) under nitrogen was added to a stirred solution of methylacrylate (10.05 g, 0.0012 M) in methanol (30 mL), during 3 hrs. The nal mixture was stirred at 0 °C for 1 h and then allowed to reach room temperature

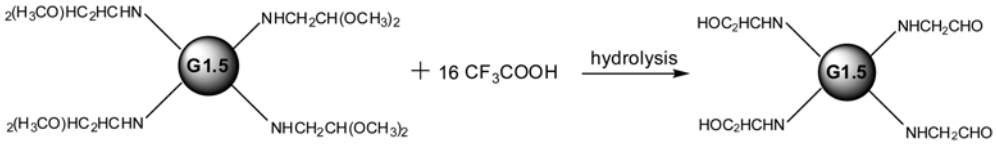

Figure 2. Synthesis pathway of aldehyde terminated dendrimer (G2): stirring at rt in methanol, 6 h.

and stirred for 3 days. The solvent was removed under reduced pressure (2 mm Hg at 40 °C) and the resultant pale yellow oil vacuum dried (2 mm Hg, 50 °C) 24 hrs to give the nal product.[23]

($^{a}CH_2{}^{a}CH_2$)[N($^{b}CH_2{}^{c}CH_2{}^{d}CO^{e}NH^{f}CH_2{}^{g}CH_2N$($^{h}CH_2{}^{i}CH_2{}^{j}CO^{k}NH^{l}CH_2{}^{m}CH_2N$($^{n}CH_2{}^{o}CH_2{}^{p}CO_2{}^{q}CH_3$)$_2$)$_2$)$_2$]$_2$: IR ($\nu$/m^{-1}): 1730 (ester C=O), 1650 (amide C=O) 3300 (NH); ^{1}H NMR ($CDCl_3$) δ_H 7.04 (12H, bt, e, k), 3.62 (48H, s, q), 3.23 (24H, bm, f, l), 2.83 (8H, bt, g), 2.69-2.76 (52H, m, a, b, c, h, i), 2.47 (32H, bt, o); 2.38 (32H, bt, n); 2.30 (16H, bt, m).

Synthesis of (G1.5) PAMAM Dendrimer with Acetal Terminated Groups. The solution of G1.5 PAMAM dendrimer with ester terminated groups (2 g, 0.71 mM) and aminoacetaldehyde dimethyl acetal (200 μL) in methanol (30 mL) was stirred at room temperature for 3 days. Then, removal of the solvent under vacuum (2 mm Hg, 45 °C, 12 hrs) gave the PAMAM dendrimer with acetal terminated groups as a viscous and adhesive product (yield; 60%) (Fig. 1).

Hydrolysis of Acetal Terminated PAMAM Dendrimer. Hydrolysis of acetal groups of acetal terminated PAMAM was carried out according to the literature with slight modification.[19] The solution of G1.5 PAMAM dendrimer with acetal terminated groups (0.5 g, 0.125 mM) and triuoroacetic acid (200 μL) in dichloromethane (15 mL) was stirred at room temperature for 48 hrs. Then, solvent was removed under vacuum (10 mmHg, 40 °C, 8 hrs). Final product is PAMAL with aldehyde terminal groups that confirmed with FTIR and ^{1}H NMR (Figs. 4, 5, 6).

($^{a}CH_2{}^{a}CH_2$)[N($^{b}CH_2{}^{c}CH_2{}^{d}CO^{e}NH^{f}CH_2{}^{g}CH_2N$($^{h}CH_2{}^{i}CH_2{}^{j}CO^{k}NH^{l}CH_2{}^{m}CH_2N$($^{n}CH_2{}^{o}CH_2{}^{p}CO^{q}NH^{r}CH_2{}^{s}CHO$)$_2$)$_2$)$_2$]$_2$: IR ($\nu$/cm^{-1}): 1725 Aldehyde C=O), 1630 (amide C=O) 3300 (NH); ^{1}H NMR ($CDCl_3$) δ_H 10.82 (16H, s, s), 5.02 (16H, bt, q), 4.72 (8H, bt, k), 4.62 (4H, bt, e), 3.69 (32H, bs, r), 3.34-3.45 (24H, bt, f, l), 2.93 (24H, bt, g, m), 2.67-2.83 (116H, bt, a, b, c, h, i, o, n).

Enzyme Immobilization. Trypsin (200 mg) was dissolved in ammonium acetate buffer (pH=8.4, 50 mM, 3 mL), then G2 PAMAL dendrimer (100 mg) was added to the solution. The mixture was stirred for 8 hrs at 4 °C, and then allowed to coupling reaction between the enzyme and the carrier. The mixture was filtered; the precipitate was washed three times with mentioned buffer to separate unbounded enzymes. Then, filtrate was collected and the residual activity was

Figure 3. Enzyme Immobilization on aldehyde terminated dendrimer**:** stirring at 4 °C in phosphate buffer.

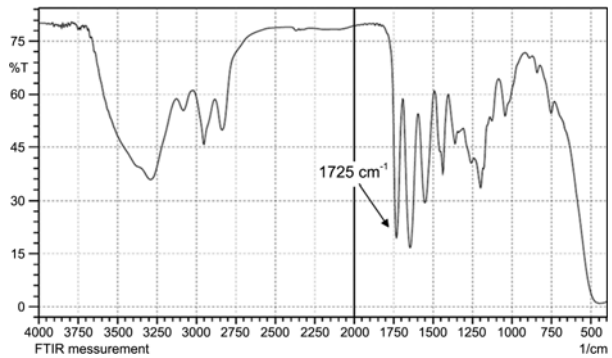

Figure 4. FT-IR spectrum of (G2) aldehyde-terminated denderimer.

determined (Fig. 3).

Enzyme Activity Assay. The activity of free and immobilized trypsin were measured with azocasein method from 30-80 °C by UV-visible spectrophotometer (2550 Shimadzu, Japan). The solution of immobilized and free trypsin (100 μL, 2 mg/mL) was mixed with 200 μL of ammonium acetate buffer (pH=8.3, 50 mM), then the enzyme catalyzed reaction was started by addition of azocasein (200 μL). After incubation at 30 °C for 60 min, the reaction was stopped by addition of 300 μL of 10% (w/v) trichloroacetic acid in methanol. The precipitate was removed by centrifugation and the absorbance of the supernatant was measured at 450 nm against a control incubated without azocasein.[24] This test was repeated for other temperatures (from 30 to 80 °C) for three times, and related data are shown in Figure 7. One unit of trypsin activity was defined as the amount of enzyme required to produce an increase in A_{450} of 0.10 in 1 h at 30 °C.[25,26]

Protein Determination. The total protein content of the samples was determined using the Bradford method with bovine serum albumin as a standard.[27]

Results and Discussion

All of the PAMAM generations were synthesized by divergent method[23] and characterized by FTIR, ^{1}H NMR and ^{13}CNMR which are suitable and efficient techniques to characterize of PAMAM dendrimers.

A FTIR spectrum of G-0.5 dendrimer showed a peak in 1740 cm^{-1} related to estric carbonyl.

^{1}H NMR spectrum presented a single peak at 3.625 ppm, a triplet peak at 2.72 ppm, a singlet peak at 2.44 ppm and a triplet peak at 2.39 ppm related to methoxy proton, 8 protons in *b* position, 4 protons in *a* position, and 8 protons in *c* position, respectively. In studying of FTIR spectrum of G1.5 with acetal terminal group, the peak in 1725 cm^{-1} confirms

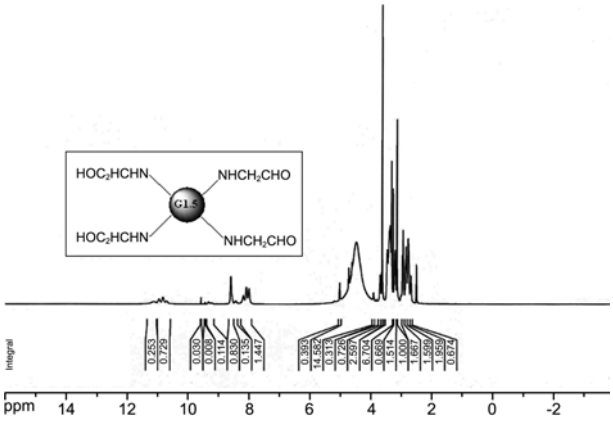

Figure 5. ^{1}H NMR spectrum of G2 dendrimer with aldehyde terminal groups.

the existence of aldehyde carbonyl group (Fig. 4), the study of ^{1}H NMR spectrum presents a single peak in 3.670 ppm related to methoxy protons and the ^{1}H NMR spectrum of G2 with acetal terminated groups shows a peak in 3.19 ppm related to methoxy protons. Investigation of ^{1}H NMR spectrum of G2 dendrimer with aldehyde terminal groups shows a peak in 10.82 ppm related to hydrogen of aldehyde (Fig. 5) and study of ^{13}C NMR spectrum of this product shows a peak in 203.319 ppm related to carbonyl of aldehyde (Fig. 6). For proving covalent attachment of trypsin and dendrimer, ^{1}H NMR was used. In comparison of ^{1}H NMR of dendrimer with dendrimer-protein bioconjugate was observed that peak of aldehyde hydrogen was disappeared. It shows that aldehyde group was reacted with enzyme by covalent bound.

Immobilized Enzyme Efficiency. The immobilization efficiency which was calculated as the ratio of activity of Immobilized enzyme to the activity of the free enzyme was found to be 46.2% which indicated that Immobilized enzyme activity decrease following immobilization of PAMAL dendrimer.

The chemical modification of enzyme during immobilization often leads to a minor modification in the enzyme conformation, resulting in loss of its catalytic activity.[24,25] It is also possible that the decreased enzyme activity for the

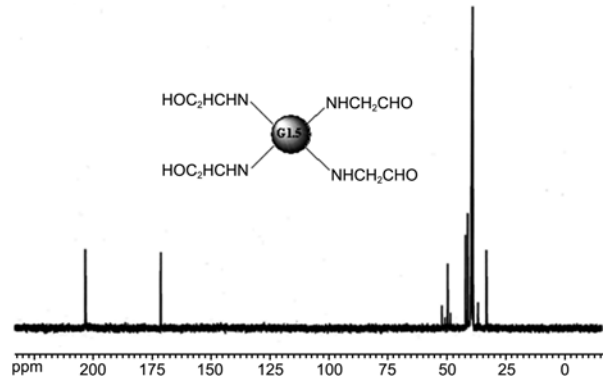

Figure 6. ^{13}C NMR spectrum of G2 dendrimer with aldehyde terminal groups.

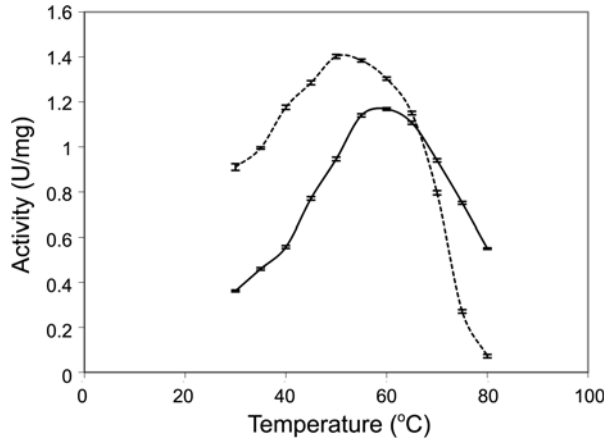

Figure 7. The activity of the free and immobilized trypsin at various temperatures (Black line for immobilized enzyme and dash line for free enzyme). The reactions were carried out in ammonium acetate buffer (50 mM, pH = 8.3) with azocasein as substrate as described in the Methods section(error bars are represented basis on standard deviations(SD) values and because of low value of SD values, error bars aren't very recognizable).

immobilized sample arises from the fact that when the enzyme molecule is attached to a carrier, its active site might be sterically blocked and, therefore, the enzyme loses its proper interaction with its substrate.

Effect of Temperature on Enzyme Activity. The effects of temperature on the relative activity of free and immobilized trypsin are shown in (Fig. 7). As expected, the enzyme activity of both free and immobilized enzymes increased as the temperature was elevated to higher values; but further increase in the temperature resulted in the reduction of the activates. Enzyme activity normally increases up to a maximum level with an increase in temperature and thereafter it decline because of denaturation of the protein. In the present study, the maximum relative activity of the free enzyme was observed at 50 ºC, whereas this value for the immobilized form was shifted to 60 ºC. Although at lower temperatures the free enzyme showed greater activity than the immobilized enzyme, the immobilization resulted in a relatively higher activity at higher temperatures.

The immobilized trypsin also showed a higher thermal stability than the corresponding free enzyme. More than 55% of the activity preserved at 80 ºC for the immobilized enzyme, but only 5% of the activity obtained for the free trypsin at 80 ºC. It seems that the dendrimer has a protective effect at high temperatures (where enzyme deactivation occurs). Therefore, the immobilization of trypsin on G2 PAMAL dendrimer, not only resulted in a relatively higher optimal temperature, but also it increased the thermal stability of the immobilized enzyme compared to the free enzyme.

The enzyme activity could be decreased, enhanced or remain unchanged following immobilization. As many common enzymatic industrial reactions are carried out at high temperatures, thermostable enzymes have attracted considerable interest in biotechnology and industry in recent years.[28] The enhancement of enzyme stability can be achiev-

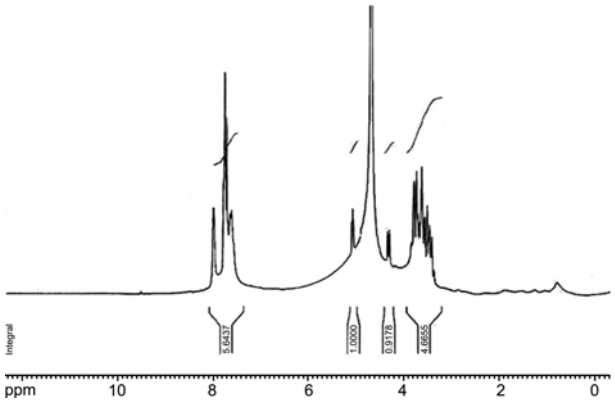

Figure 8. ^{1}H NMR spectrum of aldehyde-terminated dendrimer-trypsin bioconjugate.

ed through different strategies including immobilization technique.[29] Immobilization can sometimes increase the thermal stability of an enzyme,[30] and this property of the immobilized enzyme is considered as one of the advantages of immobilization technique in biotechnology and industrial enzymology. Immobilization of an enzyme, in particular by covalent bonding, can influence the conformational flexibility of the enzyme and causes an increase in its rigidity and, therefore, stabilizes the molecular configuration of the enzyme against alternations including those that may cause thermal deactivation.[30,31] The weak intra-molecular forces of a free enzyme can be readily disrupted at higher temperatures leading to unfolding of the protein chain; immobilization can stabilize the weak ionic forces and hydrogen bonds and subsequent increasing thermal stability of the immobilized enzyme.[32]

A higher activity for immobilized enzyme compared with the free enzyme at higher temperatures may also originate from the release of physically adsorbed trypsin molecules after buffer wash at an elevated temperature. However, it is less likely, because the free trypsin is very thermoliable at > 70 ºC (Fig. 7). It is more likely that the chemically attachment of trypsin on the dendrimer and the establishing the conformation of the enzyme against thermal denaturation serve as major factors in the enhanced stability of the immobilized trypsin at higher temperatures. This is consistent with the results obtained from the spectroscopic studies. However, further studies are required to clarify the issue.

Conclusion

Polyamidoaldehyde (PAMAL) dendrimers were synthesized using aminoacetaldehyde dimethylacetal instead of glutaraldehyde and prevent side chain crosslinking problem in producing dendrimers with terminal aldehyde group, successfully. Then, it was used as a carrier in immobilization of trypsin. Although the catalytic activity of the immobilized enzyme was lower than of the free enzyme, the immobilized enzyme showed a higher thermal stability.

Many common enzymatic industrial reactions are performed at high temperatures, and an appropriate compromise between harsh working conditions and enzyme stability has been one of the major problems in most enzymatic industrial processes.[28] Therefore, a higher thermal stability observed for the immobilized trypsin as a model protein on G2 PAMAL dendrimer could be of great value.

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