Research Report

Synthesis of Polymer-Trypsin Conjugates and their Esterase and Amidase Activities

(การสังเคราะห์และการออกฤทธิ์เป็น esterase และ amidase ของ พอลิเมอร์-ทริปซิน คอนจุเกต)

by

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PEG-trypsin conjugates

Abstract

PEGylated proteins are routinely used as therapeutics, but systematic studies of the effect of PEG molecular weight and linking chemistry on the biological activity and particularly the thermal stability of the conjugated protein are rarely made. Here, activated monomethoxypolyethylene glycol (mPEG)s (Mw 1100, 2000 and 5000 g/mol) were prepared using succinic anhydride (SA), cyanuric chloride (CC) or tosyl chloride (TC) and used to synthesise a library of trypsin conjugates. The enzyme activity (K_M, V_{max} and K_{cat}) of native trypsin and the mPEG-modified trypsin conjugates was compared using *N*-benzoyl-L-arginine *p*-nitroanilide (BAPNA) as a substrate, and their thermal stability determined using both BAPNA and *N*-α-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) as substrates to measure amidase and esterase activity respectively. The effect of conjugate chemistry on trypsin autolysis was also examined at 40 °C. PEG-trypsin conjugates containing the higher molecular weight of mPEG (5000 g/mol) were more stable than free trypsin, and the conjugate containing CC-mPEG 5000 g/mol had the best thermal stability.

Keywords: Monomethoxypolyethylene glycol (mPEG); PEG conjugates; Thermal stability; Trypsin

บทคัดย่อ

ปัจจุบันมีการใช้ PEGylated protein เพื่อการรักษาอย่างกว้างขวาง แต่ยังไม่มีการศึกษาผลของน้ำหนักโมเลกุลของ PEG และพันธะที่ใช้เชื่อม ต่อการออกฤทธิ์ทางชีววิทยาและความคงตัวต่ออุณหภูมิของ conjugated protein ในการศึกษานี้ ได้เชื่อม activated monomethoxypolyethylene glycol (mPEG) ที่มีน้ำหนักโมเลกุล 1100, 2000 และ 5000 g/mol กับ trypsin โดยใช้ตัวเชื่อมต่าง ๆ ได้แก่ succinic anhydride (SA), cyanuric chloride (CC) หรือ tosyl chloride (TC) มีการเปรียบเทียบ enzyme activity (K_m, V_{max} และ K_{cat}) ของ native trypsin กับ mPEG-modified trypsin conjugate โดยใช้ *N*-benzoyl-L-arginine *p*-nitroanilide (BAPNA) เป็น substrate และศึกษาความคงตัวที่อุณหภูมิต่าง ๆโดยใช้ทั้ง BAPNA และ *N*-α-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) เป็น substrate เพื่อวัดการออกฤทธิ์เป็น amidase และ esterase ตามลำดับ นอกจากนี้ ยังศึกษาผลของโครงสร้างเคมีของ conjugate ต่อ autolysis ของ trypsin ที่ 40°C ผลการศึกษาพบว่า PEG-trypsin conjugate ที่ประกอบด้วย mPEG ที่มีน้ำหนักโมเลกุลสูง (5000 g/mol) มีความคงตัวกว่า trypsin และ conjugate ที่มี CC-mPEG 5000 g/mol มีความคงตัวต่ออุณหภูมิได้ดีที่สุด

Introduction

The biotechnology revolution has produced many novel peptides and proteins that have become important new drugs. More than 80 are nowmarketed in the United States, and 350 are undergoing clinical trials (reviewed in Harris and Chess, 2003). However, protein drugs also possess several shortcomings that limit their usefulness. These include premature degradation due to susceptibility to destruction by proteolytic enzymes, short circulating half-life, low solubility, rapid kidney clearance of lower molecular weight proteins and potential immunogenicity. Conjugation of proteins and peptides to natural or synthetic polymers has shown the ability to solve many of these problems (reviewed in Harris and Chess, 2003; Pasut and Veronese, 2007).

Although a variety of polymers have been used for protein conjugation, polyethylene glycol (PEG) has been most popular due to its excellent water-solubility, and it is known to be safe being FDA approved for use in injectable, topical, rectal and nasal formulations. PEG is synthesised by anionic ring polymerisation of ethylene oxide using methanol or water as initiator to give a linear polyether that can be modified to ensure a single terminal reactive functional group e.g. monomethoxy PEG (mPEG). This enables protein modification without risk of cross-linking (reviewed in Pasut and Veronese, 2007; Duncan, 2003; Greenwald et al., 2003; Harris and Chess, 2003). Moreover, PEG polymerisation can be controlled to produce a wide range of discreet molecular weights (<5000–50,000 g/mol) with low polydispersity (Mw/Mn from <1.01 to 1.1 for higher PEGs of Mw ~50,000 g/mol). The high hydration and flexibility of the PEG chain enables a reduction in antigenicity of proteins to which it is bound, and careful choice of PEG molecular weight enables fine tuning of plasma pharmacokinetics and route of elimination (by renal and hepatic pathways) of specific peptides and proteins to suit their pharmacodynamic requirements.

Despite the large number of studies on PEG-protein modification, including the emergence of novel linking chemistry (Brocchini et al., 2006), there has been little attempt to systematically study the effect of PEG molecular weight and linking chemistry on protein properties such as thermal stability. Therefore, the aims of this study were to compare the enzyme activity (kinetic parameters), the thermal stability and autolysis of a library of PEG-modified trypsin conjugates synthesized using PEGs of different molecular weights (Mw 1100, 2000 and 5000 g/mol) and different linking chemistries. Trypsin (from bovine pancreas) was chosen as a convenient model protein. It is a pancreatic serine protease, composed of a single polypeptide chain of 223 amino acid residues, and it displays a narrow substrate specificity hydrolysing l-lysyl and l-argininyl bonds of polypeptides (amidase activity; Walsh, 1970 and esterase activity; Bergmeyer et al., 1974). As the covalent attachment of *p*-nitrophenyl chloroformate (NPC) activated mPEG to bovine pancreatic trypsin increased thermal stability (Gaertner and Puigserver, 1992; Zhang et al., 1999) the PEG-trypsin conjugates were synthesised using succinic anhydride (SA), cyanuric

chloride (CC) or tosyl chloride (TC) to activate PEG (Scheme 1). The enzyme activities (K_M, V_{max} and K_{cat}) were determined by using *N*-benzoyl-L-arginine *p*-nitroanilide (BAPNA) and *N*-α-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) as substrates for amidase and esterase activity, respectively.

Materials and methods

Materials

mPEGs of molecular weight 1100, 2000 and 5000 g/mol, succinic anhydride and 4-dimethylaminopyridine (DMAP) were from Fluka (Switzerland). Bovine pancreatic trypsin (EC 3.4.21.4), cyanuric chloride, tosyl chloride, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), 2,4,6-trinitrobenzene sulfonic acid (TNBS), BAPNA, BAEE, and the Coomassie blue for protein assay were from Sigma (Germany). All other reagents and chemicals were of analytical grade.

Methods

Preparation of activated mPEG

Succinoylated mPEG (SA-mPEG) (Scheme 1a). mPEG (1100, 2000 or 5000 g/mol; 1 mmol) was dried by azeotropic distillation using toluene and then dissolved in anhydrous dimethylformamide (DMF, 10 ml). Succinic anhydride (4 mmol) was added, followed by DMAP (4 mmol). The mixture was stirred overnight at room temperature, the resulting polymer was then precipitated using ether, recrystallized twice from CH_2Cl_2/ether (1:40) and characterised by TLC (BuOH/AcOH/H_2O, 4:1:1) (Zalipsky et al., 1983), FTIR and ^1H NMR. The acid groups were quantified by titration against standardized NaOH solution using bromothymol blue as an indicator (Hreczuk-Hirst et al., 2001).

Cyanurate mPEG (CC-mPEG) (Scheme 1b). Cyanuric chloride (3 mmol) was dissolved in CH_2Cl_2 containing anhydrous sodium sulphate. mPEG (1100, 2000 or 5000 g/mol; 1 mmol) was added and the mixture was stirred overnight at room temperature. The solution was then filtered, and ether was added slowly with stirring. The finely divided precipitate was collected on a filter and re-dissolved in CH_2Cl_2. This precipitation and filtration process was repeated several times until the ether was free of residual cyanuric chloride as determined by TLC ($CHCl_2$/MeOH, 7:1) following the method of Schiavon et al. (2004). The product was characterised by FTIR and ^{13}C NMR.

Tosylate mPEG (TC-mPEG) (Scheme 1c). Tosyl chloride (3 mmol) was dissolved in CH_2Cl_2 containing anhydrous sodium sulphate. mPEG (1100, 2000 or 5000 g/mol; 1 mmol) and

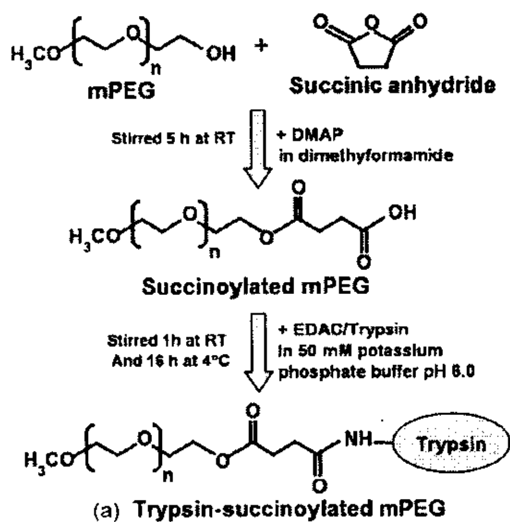
triethylamine (1 mmol) was added and the mixture was stirred overnight at room temperature. The solution was filtered, and ether was added slowly with stirring. The resulting white powder was collected on a filter and re-dissolved in CH_2Cl_2, washed several times until the ether was free of residual tosyl chloride as determined by TLC ($CHCl_3$/MeOH, 9:1). The product was characterised by FTIR and ^{1}H NMR.

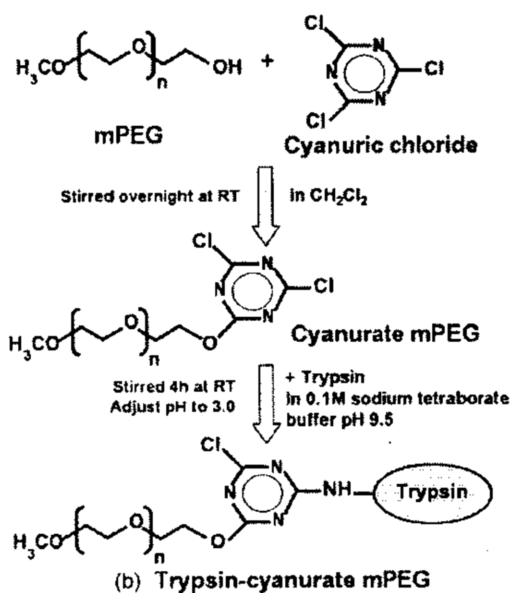
PEGylation of trypsin Trypsin was modified with the activated mPEGs synthesized above as follows.

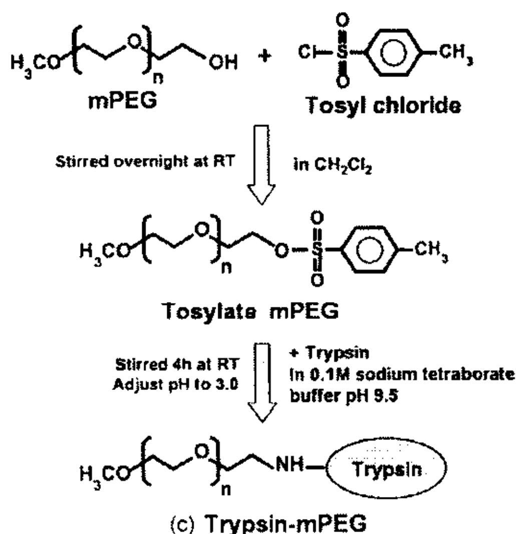
Trypsin-succinoylated mPEG (Trypsin-SA-mPEG) (Scheme 1a). EDAC was added to the reaction mixtures containing trypsin dissolved in 10 ml of 50mM potassium phosphate buffer, pH 6.0, and succinoylated mPEGs of molecular weight 1000, 2000 and 5000 g/mol using a molar ratio of trypsin: succinoylated mPEGs (1:20, 1:40 and 1:60). The solution was stirred for 1 h at room temperature and then for16h at 4 °C. It was then dialysed at 4 °C for 48 h against water using a dialysis membrane of molecular weight cut-off 12,400 and the product was finally lyophilized (Fernandez et al.,2003).

Trypsin-cyanurate mPEG (Trypsin-CC-mPEG)(Scheme 1b). Cyanurate mPEG was reacted with trypsin solubilised in 0.1Msodium tetraborate buffer (pH 9.5, 2 mg/ml). As above a molar ratio of trypsin: cyanurate mPEGs of 1:20, 1:40 and 1:60 was used. The resulting mixture was stirred at room temperature for 4 h, and then brought to pH 3.0, and dialysed at 4 °C as above and the product was lyophilised (Abuchowski and Davis, 1979).

Trypsin-tosylated mPEG (Tryp-TC-mPEG or TrypsinmPEG) (Scheme 1c). Tosylated mPEG was reacted with trypsin solubilised in 0.1Msodium tetraborate buffer (pH 9.5, 2 mg/ml) using the same molar ratios as given above. The resulting mixture was stirred at room temperature for 4 h, the solution then brought to pH 3.0, and dialysed at 4 °C as above and the product was lyophilised (Gaertner and Puigserver, 1992).







Scheme 1 Reaction scheme for synthesis of (a) trypsin-succinoylated mPEG conjugates; (b) trypsin-cyanurate mPEG conjugates; (c) trypsin-mPEG conjugates

Characterisation of the trypsin conjugates

In all cases, the Bradford protein assay was used to determine the total protein content of the conjugates. The extent of the protein modification was also estimated by determining the number of remaining free amino groups using the TNBS assay as described by Fields (1971). Briefly, sample (20 μl) was added to 0.1M sodium tetraborate buffer in 0.1M NaOH (0.5 ml) and the volume was made up to 1 ml. Then, TNBS solution (20μl) was added and the solution was rapidly mixed. After 5 min the reaction was stopped by adding 0.1M sodium phosphate buffer containing 1.5M sodium sulfite (2.0 ml), and the absorbance at 420 nm was determined. A blank was prepared similarly without sample.

Determination of trypsin (native and conjugate) activity using BAPNA

Amidase activity of native and the PEGylated-trypsin conjugates was measured using BAPNA as a substrate (Murphy and O'Fagain, 1996). Briefly, 4.2mM BAPNA (18.2 mg dissolved in 1ml DMSO and added to 9ml of 0.1M Tris–HCl, pH 8.2 containing 20mM $CaCl_2$) (400μl) and 0.1M Tris–HCl, pH 8.2 containing 20mM $CaCl_2$ (580μl) were added to a 1 ml cuvette. Then, 20μl of free trypsin or PEG-trypsin conjugate (10μg protein-equiv. in 0.1M Tris–HCl, pH 8.2 containing 20mM $CaCl_2$) was added and the solution was thoroughly mixed. The mixture was then incubated in the water bath at 30 °C for 10 min. The absorbance was read using a Shimadzu UV 1601 Spectrometer (Kyoto, Japan) at 410 nm, and one unit of activity defined as the amount of enzyme that hydrolyses 1 μmol BAPNA/min. The trypsin activity was calculated using a range of substrate concentrations (0.4–2.0 mM) and a molar extinction coefficient 8800M^{-1} cm^{-1} for free *p*-nitroaniline (PNA). The reaction volume was always 1.0 ml. To calculate the kinetic parameters, a Lineweaver–Burk plot (Erlanger et al., 1961) was constructed as follows:

Unit of activity (unit/mg) = (ΔAbs_{410nm} x 1000 x 1.0)/(8800 x mg protein)

Determination of trypsin (native and conjugate) activity using BAEE

Esterase activity of native and PEGylated-trypsin conjugates was determined at 25 °C using BAEE as substrate. Briefly, 20 μl of free trypsin or PEG-trypsin conjugate (10 μg protein-equiv.) was added to a cuvette containing 3 ml of 0.25 mM BAEE in 67 mM sodium phosphate buffer, pH 7.6, then immediately mixed thoroughly by inversion. The increase in absorbance at 253 nm was measured for approximately 10 min and the trypsin activity was calculated for a range of substrate concentrations 0.025–0.25 mM. One unit of esterase activity was defined as the amount of enzyme that hydrolyses 1.0 mmol of BAEE/min at 25 °C (Bergmeyer et al., 1974) as follows:

Unit of activity (unit/mg) = [(ΔAbs_{253nm} test — ΔAbs_{253nm} blank)1000 x 3.02] /mg protein

Evaluation of the thermal stability of PEG-trypsin conjugates

To test the thermal stability, native trypsin and the PEGylated conjugates were incubated in aqueous buffer (1.67mM of 0.1M Tris–HCl, pH 8.2 containing 20mM $CaCl_2$ for amidase activity and 0.25mM of 67mM sodium phosphate buffer, pH 7.6 for esterase activity) for 10 min at temperatures between 30 and 70 °C. Then the residual trypsin for amidase or esterase activity was assayed using BAPNA or BAEE as described above. The activities of samples were compared to that at 30 °C (Zhang et al., 1999; Fernandez et al., 2002, 2003).

Evaluation of autolysis of PEG-trypsin conjugates

Native trypsin and the PEGylated-trypsin conjugates were incubated at 40 °C in aqueous buffer (0.1M Tris–HCl, pH 8.2 containing 20mM $CaCl_2$ for amidase activity and 67mM sodium phosphate buffer, pH 7.6 for esterase activity) for 180 min. Aliquots of free trypsin or PEG-trypsin conjugate (10 μg protein-equiv.) (20 μl) were taken at different times and assayed for amidase or esterase activity using BAPNA or BAEE as described above.

Results and discussion

Synthesis and characterisation of the activated mPEG intermediates and PEG-trypsin conjugates

The succinoylation of mPEGs of different molecular weight was monitored by TLC. No free succinic anhydride was detected in the product, and the content of acid groups was 87–98% by titration. FTIR of SA-mPEG showed disappearance of the characteristic of OH signals of mPEG at 3461 cm^{-1}, with the characteristic ester (1731 cm^{-1}) and carboxyl group (COOH) of succinoyl moiety (1647 cm^{-1}). ^{1}H NMR confirmed the succinoylated mPEG characteristic peaks through the aliphatic protons of succinoyl moiety at 2.5–2.7 ppm (4H) and multiplet band of PEG protons at 3.5–4.2 ppm. The FTIR spectra of CC-mPEG showed disappearance characteristic of OH moiety of mPEG at 3470 cm^{-1}, with a C=N characteristic peak at 1700 cm^{-1} and the skeleton vibration of the 1,3,5-triazine ring at 803 and 1500 cm^{-1}. ^{13}C NMR showed 1,3,5-triazine carbon ring at 172.1–174.2 ppm and carbon skeleton of PEG at 69.7–72.7 ppm. Similarly for the TC-mPEG the FTIR spectra showed disappearance characteristic of OH moiety of mPEG at 3413 cm^{-1} with, in this case, the characteristic SO_2 (1700 cm^{-1}) and the aromatic (at 1398 and 1176 cm^{-1}) peaks. ^{1}H NMR confirmed aromatic protons at 7.4–7.8 ppm and multiplet band of PEG protons at 3.2–3.7 ppm (results not shown). These activated mPEG intermediates were then used to prepare the library of trypsin conjugates. All the molecular weight PEGs produced comparable levels of amino groups substitution of trypsin (Table 1). When experiments were carried out to ascertain the optimum ratio between trypsin:activated mPEG (reactions at molar ratios of 1:20,

1:40 and 1:60), a reaction ratio of 1:20 gave an adequate degree of substitution; increased amounts of the activated mPEG concentration did not show a higher degree of substitution. The TNBS assay indicated that native trypsin had 11 accessible amino groups compared to the primary structure trypsin from bovine pancreas which contains 14 lysine amino groups (Walsh, 1970). After modification, it appeared that 4–9 mPEG molecules were covalently bound depending on the reaction conditions and specific mPEG used. For all PEG molecular weights, the degree of modification was achieved using TC-mPEG > CC-mPEG > SA-mPEG (Table 1).This is probably due to the fact that tosyl chloride is a better leaving group for the conjugation condition used.

Table 1 Estimated number of amino groups substituted in trypsin†

Conjugate	Molar ratios of trypsin and PEG	mPEG 1100††	mPEG 2000††	mPEG 5000††
Tryp-SA-mPEG	1:20	4.4 ± 0.7	4.6 ± 0.1	5.4 ± 0.1
Tryp-SA-mPEG	1:40	5.3 ± 1.2	3.2 ± 0.2	4.8 ± 0.1
Tryp-SA-mPEG	1:60	5.2 ± 0.8	2.6 ± 0.2	5.2 ± 0.1
Tryp-CC-mPEG	1:20	8.5 ± 0.4	6.9 ± 0.1	7.6 ± 0.2
Tryp-CC-mPEG	1:40	8.6 ± 0.1	5.2 ± 0.5	6.8 ± 0.1
Tryp-CC-mPEG	1:60	8.5 ± 0.3	4.0 ± 0.3	4.8 ± 0.8
Tryp-TC-mPEG	1:20	9.1 ± 0.1	9.7 ± 0.1	8.5 ± 0.4
Tryp-TC-mPEG	1:40	8.9 ± 0.1	9.5 ± 0.1	7.0 ± 0.2
Tryp-TC-mPEG	1:60	9.0 ± 0.1	8.9 ± 0.1	7.0 ± 0.8

† It should be noted that native trypsin has ~ 11 amino groups in the TNBS assay

†† Data show mean ± SD (n = 3)

Enzyme activity of trypsin and PEG-trypsin conjugates measured using BAPNA

The kinetic parameters obtained for both native trypsin and the PEGylated-trypsin conjugates (measured using BAPNA as substrate) are summarised in Table 2. All conjugates had lower activity than native trypsin. The conjugates synthesised using higher molecular weight PEGs displayed much lower K_M values indicating a higher affinity for the binding site of trypsin. Increasing molecular weight of mPEGs resulted in a decrease in the K_M and V_{max} values seen. These results were in contrast to the observation of Gaertner and Puigserver (1992) as they found that all conjugates had a higher amidase activity (in terms of percentage) than trypsin. In addition, the influence of the linkers on the enzyme efficiency of the conjugates as reported in Table 2 for K_{cat}/K_M values should be noted. Modification of trypsin with cyanurate mPEG increased this catalytic parameter more than seen for the other conjugates. The trypsin conjugate prepared using succinoylated mPEG displayed a higher rate of hydrolysis (mM/min) than other

conjugates at 30 °C (Fig. 1). This was likely due to the fact that it contained fewer mPEG chains than the other conjugates resulting in lower steric hindrance of the trypsin active site (Veronese, 2001).

Table 2 Enzyme activity. Kinetic parameters of native and PEG-trypsin conjugates[†]

Conjugate	Residual activity (%)	K_m (mM)	V_{max} (min)	K_{cat} (s^{-1})
Trypsin	100	0.88 ± 0.09	0.177 ± 0.002	6.1 ± 0.8
Tryp-SA mPEG 1100	96.2 ± 2.0	1.15 ± 0.16	1.047 ± 0.389	6.6 ± 0.6
Tryp-SA mPEG 2000	76.8 ± 9.5	0.93 ± 0.09	0.457 ± 0.061	5.3 ± 0.8
Tryp-SA mPEG 5000	65.2 ± 8.3	0.79 ± 0.03	0.206 ± 0.026	4.0 ± 0.5
Tryp-CC mPEG 1100	17.5 ± 0.9	0.83 ± 0.09	0.104 ± 0.025	1.1 ± 0.2
Tryp-CC mPEG 2000	49.0 ± 9.8	0.45 ± 0.13	0.089 ± 0.017	2.7 ± 0.6
Tryp-CC mPEG 5000	92.9 ± 8.7	0.34 ± 0.07	0.040 ± 0.004	4.7 ± 0.5
Tryp-TC mPEG 1100	19.9 ± 2.4	0.71 ± 0.11	0.160 ± 0.014	1.2 ± 0.1
Tryp-TC mPEG 2000	68.5 ± 0.3	0.66 ± 0.02	0.063 ± 0.001	4.1 ± 0.1
Tryp-TC mPEG 5000	67.6 ± 0.6	0.48 ± 0.06	0.036 ± 0.010	3.4 ± 0.5

[†]In all cases the conjugates were prepared using a molar ratio of 1:20

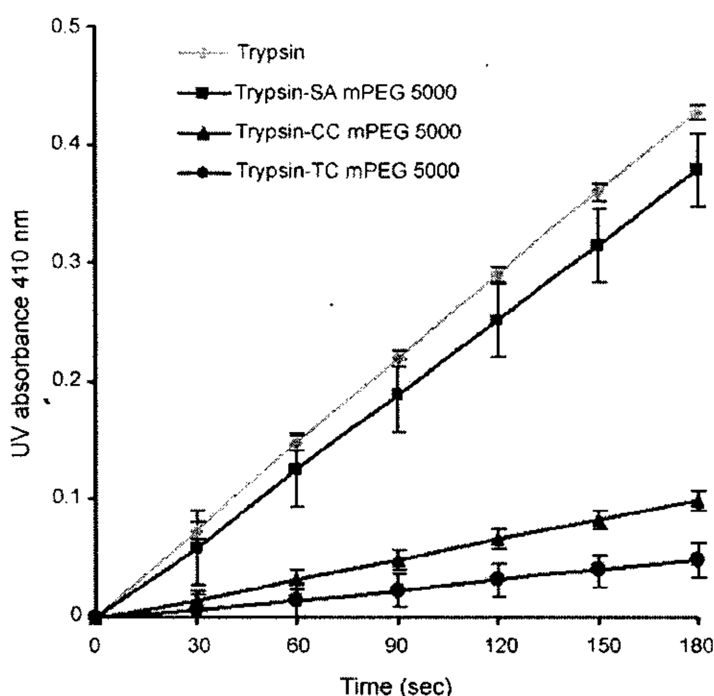


Fig. 1. Comparison of the activity of native and PEG-modified trypsin conjugates. The PEG used had a molecular weight of 5000 g/mol. In each case 10 mg/ml protein (trypsin) equivalent was used (mean±S.D.; n=3).

Thermal stability and autolysis of trypsin and PEG-trypsin conjugates

All mPEG-modified trypsins showed increased thermal stability compared to native trypsin (Figs. 2 and 3). This was true at all temperatures studied (except 70 ˚C) and when both BAPNA (Fig. 2) and BAEE (Fig. 3) were used as substrates. The highest activities were found when using the PEG with molecular weight of 5000 g/mol in all cases. This may be due to greater protection given by the higher molecular weight polymer chain; the hydrophilic PEG chain can swell and wrap around trypsin. Moreover, the PEG chain can form a highly hydrogen-bonded structure around the trypsin molecule (Gaertner and Puigserver, 1992; Zhang et al.,1999).

The time course for autolysis for native and modified trypsin at 40 ˚C was determined using both BAPNA and BAEE as substrates (Figs. 4 and 5 respectively). The half-life (*t*1/2) of the PEG-trypsin conjugates was increased up to 4 fold compared to native trypsin (Table 3), and trypsin-modified with mPEG 5000 displayed the highest percentage retention of residual activity using both substrates and for all types of linker used. The values of residual activity measured using BAPNA as substrate were: native trypsin (13%), trypsin-SA-mPEG 5000 (37%), trypsin- CC-mPEG 5000 (50%) and trypsin-TC mPEG 5000 (39%) respectively. Stability was thus in the order CC > TC > SA, and moreover the same result was observed when using BAEE as substrate (residual activity 2, 8, 75 and 38% respectively).

All the mPEG-trypsin conjugates were less prone to autolysis compare to the native trypsin and they showed similar autolysis patterns. As the molecular weight of mPEG decreased the conjugates were less stable. This increased stability might be explained by specific modification of lysine residues (remembering the substrate specificity of trypsin) as this would inherently be expected to protect against autolysis, and/or the fact that the mPEG chains attached on the enzyme surface would anyhow cause non-specific steric hindrance (Murphy and O'Fagain, 1996). However, the observation that the higher molecular weight PEGs produced greater trypsin stability suggest a major role for the latter mechanism. The increasing of thermal stability of modified trypsins could also result from the formation of a highly hydrogen-bonded structure, with polymer wrapped around the protein (Gaertner and Puigserver, 1992; Zhang et al.,1999).

In the context of therapeutic protein formulation development, the active ingredient must be stable during formulation manufacture and for the shelf-life of the product. As thermal stability can be a good predictor of activity it is important to note that trypsin-modified with mPEG 5000 showed better stability compared to the native trypsin when tested at high temperature (Fig. 2). This study therefore underlines that, not only is the choice of PEG molecular weight and linking chemistry important in terms of retained biological activity of the protein, it may have an important impact on issues relating to formulation development.

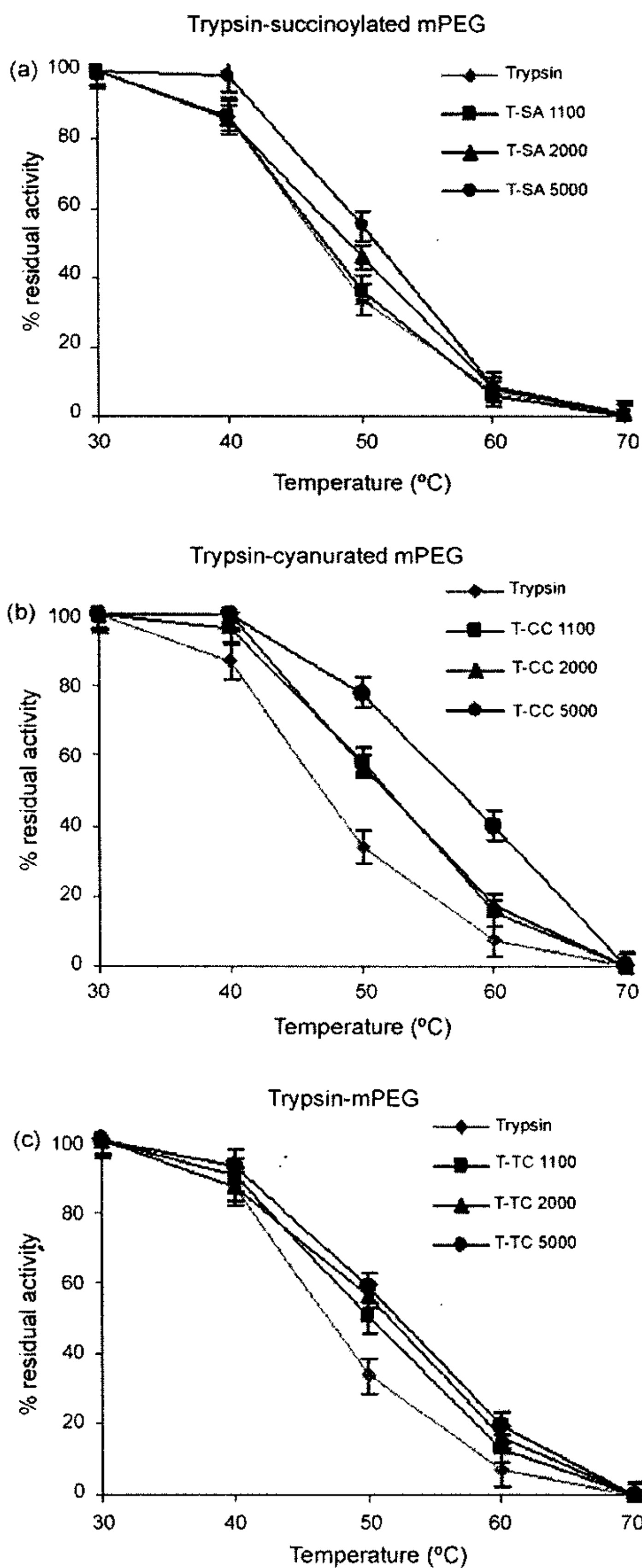


Figure 2 Effect of temperature on the activity of native and PEG-modified trypsin conjugates measured using BAPNA as a substrate (mean±S.D.; n = 3).

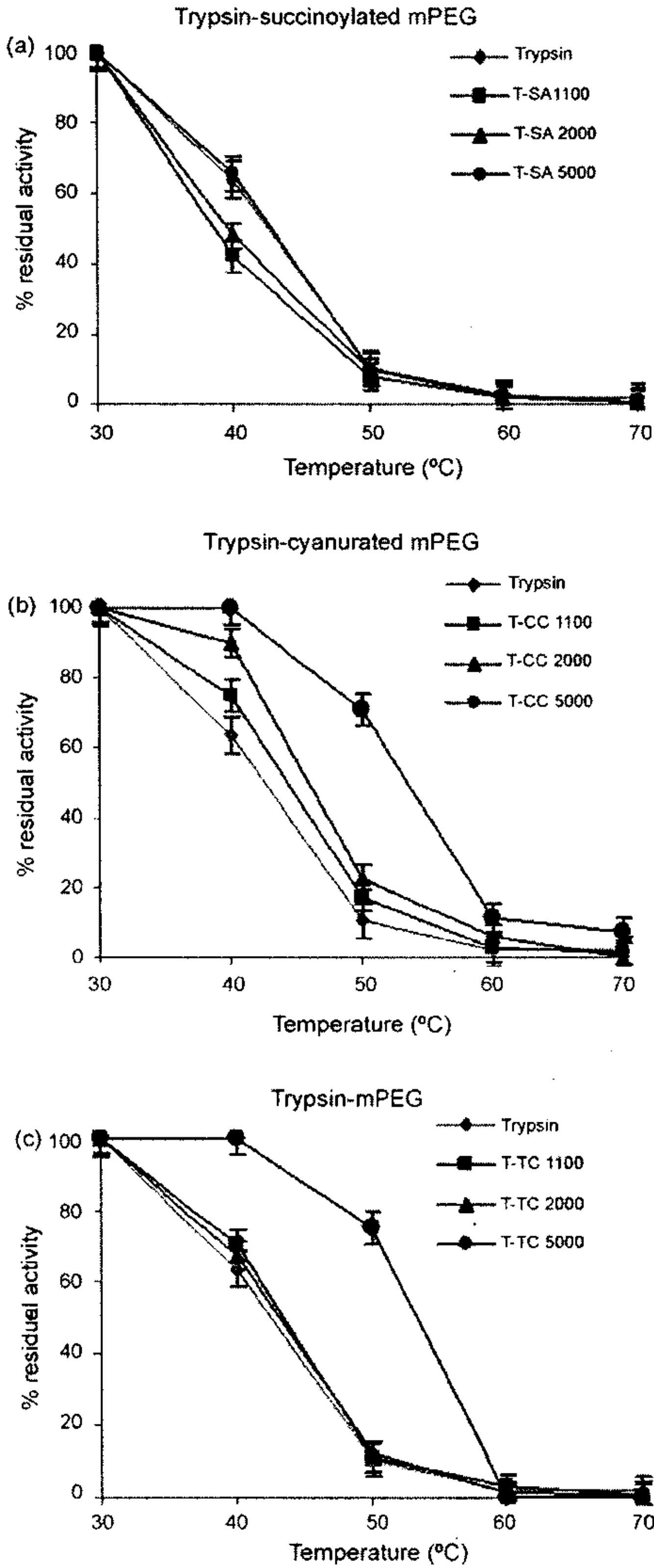


Figure 3 Effect of temperature on the activity of native and PEG-modified trypsin conjugates measured using BAEE as a substrate (mean±S.D.; n = 3).

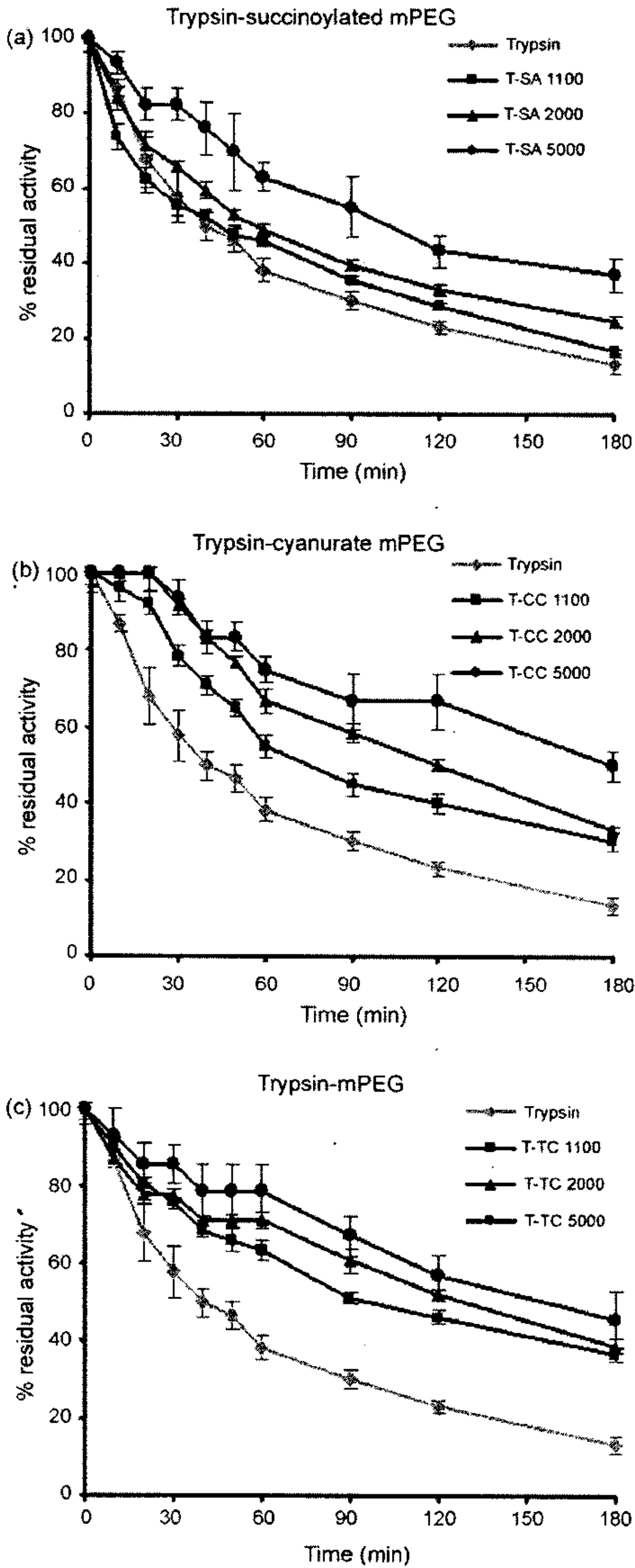


Figure 4 Autolysis of native and PEG-modified trypsin conjugates measured using BAPNA as a substrate (mean±S.D.; $n = 3$).

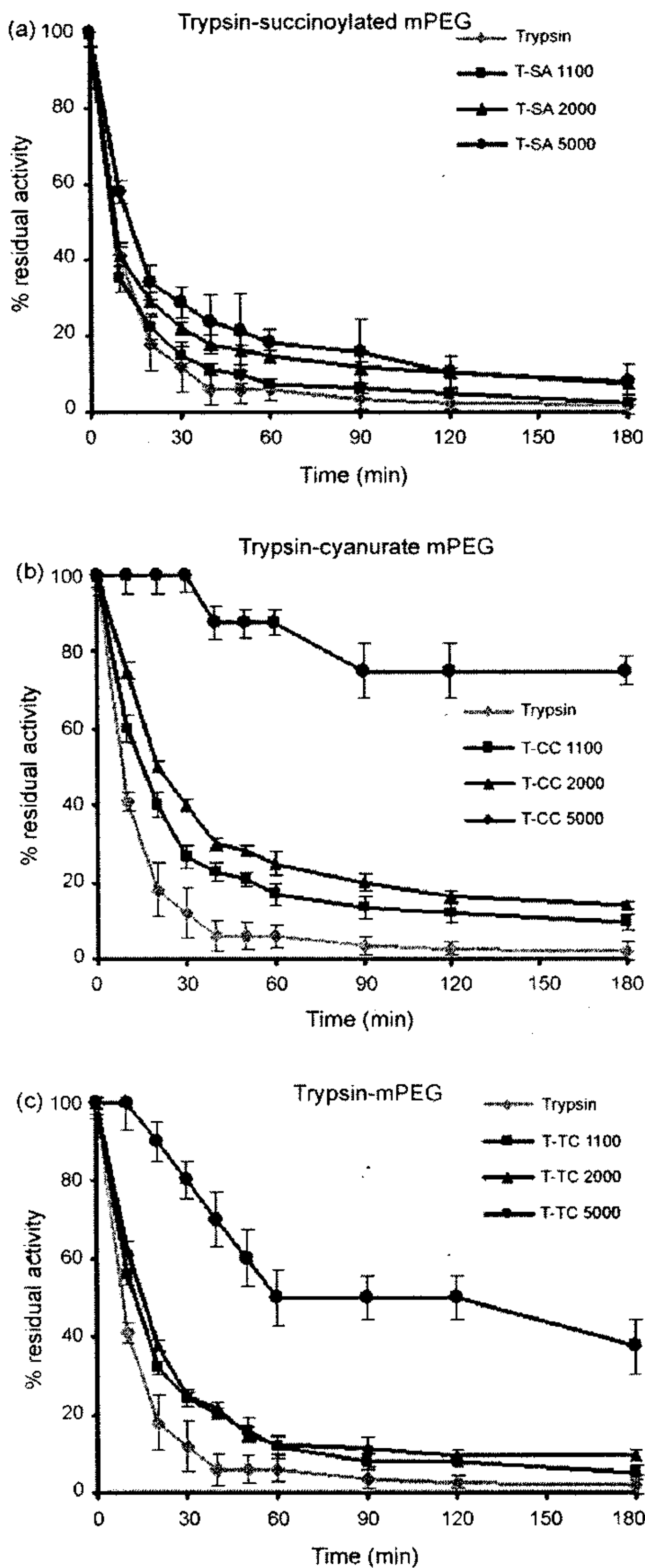


Figure 5 Autolysis of native and PEG-modified trypsin conjugates measured using BAEE as a substrate (mean±S.D.; n = 3).

Table 3 Half-life of native and PEG-trypsin conjugates[†]

Conjugate	Substrate[††]							
	BAPNA				BAEE			
	Trypsin	mPEG 1100	mPEG 2000	mPEG 5000	Trypsin	mPEG 1100	mPEG 2000	mPEG 5000
Trypsin	41 ± 1	-	-	-	8 ± 1	-	-	-
Tryp-SA-mPEG	-	45 ± 1	56 ± 1	102 ± 1	-	8 ± 1	9 ± 1	13 ± 1
Tryp-CC-mPEG	-	73 ± 1	119 ± 2	180 ± 2	-	15 ± 1	20 ± 1	>180
Tryp-mPEG	-	95 ± 2	127 ± 3	157 ± 3	-	12 ± 1	15 ± 1	60 ± 1

[†]Experiments were conducted at 40 °C and the hydrolysis of BAPNA and BAEE was used to measure residual trypsin activity

[††]Data represent $t_{1/2}$ min (mean ± SD) (n=3)

Conclusions

In this study, trypsin was successfully conjugated with mPEG of molecular weight 1100, 2000 and 5000 g/mol. Although chemical modification of mPEG to trypsin could decrease activity of trypsin, but the modified trypsin conjugates containing the higher molecular mass mPEG showed higher affinity to binding site of enzyme with substrate, higher thermal stability, more stable against autolysis and had an increased $t_{1/2}$ compared to the native enzyme. The trypsin-conjugated mPEG 5000 having cyanurate linker demonstrated the best thermal stability.

Acknowledgements

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สำนักทรัพยากรการเรียนรู้คุณหญิงหลง อรรถกระวีสุนทร

β-cyclodextrin- and PAMAM dendrimer-trypsin conjugates

Abstract

Bovine pancreatic trypsin conjugates were first synthesized using succinoylated β-cyclodextrin and succinoylated PAMAM dendrimer. Their conjugates had a trypsin content of 72 and 50 wt %, respectively. The trypsin activity of conjugates measured by using *N*-α-benzoyl-*D*, *L*-arginine-*p*-nitroanilide hydrochloride (BAPNA) as substrate was decreased by polymer conjugation. The kinetic parameters (K_M, V_{max} and K_{cat}) were also calculated. The β-cyclodextrin-trypsin conjugate was more stable than native trypsin and PAMAM-trypsin conjugate at all temperatures between 30-70°C. It also exhibited better thermal stability in the autolysis assays at 40°C.

Keywords— β-cyclodextrin, PAMAM dendrimer, polymer-protein conjugates, thermal stability

บทคัดย่อ

trypsin conjugates ถูกสังเคราะห์ขึ้นโดยใช้ succinoylated β-cyclodextrin และ succinoylated PAMAM dendrimer ปริมาณ trypsin ที่มีอยู่ใน conjugate คือ 72 และ 50% โดยน้ำหนัก ตามลำดับ การวัดค่าการออกฤทธิ์ของ trypsin โดยใช้ *N-α*-benzoyl-*D, L*-arginine-*p*-nitroanilide hydrochloride (BAPNA) เป็น substrate พบว่า การออกฤทธิ์ของ trypsin conjugate มีค่าลดลงภายหลังเชื่อมต่อกับพอลิเมอร์ การศึกษานี้ได้รายงานค่าจลนศาสตร์ต่าง ๆที่คำนวณได้ (ได้แก่ K_M, V_{max} และ K_{cat}) β-cyclodextrin-trypsin conjugate มีความคงตัวมากกว่า trypsin และ PAMAM-trypsin conjugate ที่อุณหภูมิระหว่าง 30-70°C และยังมีความคงตัวต่อ autolysis ที่อุณหภูมิ 40°C ด้วย

Introduction

The use of enzymes as therapeutic agents is often limited by the low resistance to proteolytic enzyme, short circulating half-life and immunogenicity of these proteins [1]. During the last few decades, several efforts have been devoted to the development of new methodologies for increasing functional stability of protein without affecting their biological behavior. Polymer conjugation, especially using PEG has been broadly used to reduce their immunogenicity and improve both the resistance to proteolytic degradation and thermal stability [2]. Our recent studies, a series of PEG-trypsin conjugates were synthesized using PEGs of different molecular weights and with different linking chemistries. The effect of chemical compositions on bioactivities of the conjugated enzyme, its autolysis and its thermal stability were studied. It was found that both molecular weight of PEG and the linkers play important roles on their activity and stability [3].

Historically, carbohydrate compounds have been extensively used as modifying agents for enzymes. β-cyclodextrin (β-CD, 1,135 g/mol) is a cyclic non-reducing α-(1-4)-linked D-glucopyranose composed of 7 glucose units in the chair conformation. The structure of these remarkable molecular receptors resembles a truncated annular cone with a central cavity, which is hydrophobic in nature and has the appropriate size to include a wide variety of hydrophobic guest compounds [4]. Previous study reported that amino derivatives of β-CD can be coupled to aspartic and glutamic acid residues of proteins under mild conditions [5]. β-CD was conjugated to many enzymes such as trypsin [5-7], α-chymotrypsin [8] and oxytocin [9]. Therefore, β-CD was chosen in this study and modified with a different linker using succinoylation before conjugation to amino group of trypsin.

Polyamidoamine (PAMAM) dendrimer is another interesting class of drug carriers. It has well-define structure and consists of a central core and branched monomers. It has a globular shape and a large number of end groups. The ideal dendrimer should exhibit high aqueous solubility and .drug-loading capacity, biodegradability, low toxicity, favorable retention and biodistribution characteristics, specificity, and appropriate bioavailability [10]. PAMAM dendrimer can be classified by the surface or terminal groups such as amine (-NH2), carboxyl (-COOH) and hydroxyl (-OH) groups [11]. PAMAM-NH_2 and PAMAM-OH were widely used for conjugation to many drugs such as 5-ASA [12], ibuprofen [13], methotrexate [14], methylprednisolone [15], paclitaxel [16], Penicillin V [17], propanolol [18]. However, the use of this polymer for conjugation to enzymes has not been reported. PAMAM-OH, G3 (6,941 g/mol) was chosen to bound with trypsin in this study because: PAMAM-OH has many hydroxyl group similar to β-CD.

Trypsin is widely used as a model protein. It is a well-studied serine protease involved in the digestion process of mammals that cleaves specific peptide bonds having a lysine or arginine residue [19]. Therefore, the aim of this study was to examine the effect of β-CD and PAMAM dendrimer on activity, autolysis and thermal stability of trypsin in conjugation system.

Materials and methods

Materials

β-cyclodextrin was purchased from Fluka (USA) and PAMAM dendrimer (-OH) was from Aldrich (USA). *N*-Hydroxysulfosuccinimide sodium salt (sulfo-NHS) was purchased from Fluka (Switzerland). Bovine serum albumin (BSA) was from Sigma (Switzerland). Trypsin from bovine pancreatic, *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) and N_{α}-benzoyl-*D, L*-arginine-*p*-nitroanilide hydrochloride (BAPNA) were supplied by Sigma (UK). All chemicals used in this study were analytical grade.

Methods

Succinoylation of polymers

β-cyclodextrin (0.5 g, 0.38 mmol) and succinic anhydride (0.5 g, 5 mmol) were dissolved in anhydrous pyridine (10 ml) for 4 h. The succinoylated β-cyclodextrin was then precipitated by addition of 40 ml isopropyl alcohol. The precipitate was then washed 3 times with 10 ml isopropyl alcohol [20] (Figure 1a).

PAMAM dendrimer (1 mmol) was dissolved in DMF (5 ml) and succinic anhydride (32 mmol) was added followed by DMAP (32 mmol). The reaction mixture was purged with N_2, sealed and stirred overnight at 50°C, under N_2. It was then poured into vigorously stirred diethyl ether (1 L) and stirred for 10 h. The ether was removed by filtration under vacuum and the remaining solid was dissolved in minimum amount of distilled water, poured into a dialysis membrane (molecular weight cut-off 3,500 g/mol) and dialyzed against distilled water for 48 h. The resulting solution was freeze-dried to yield succinoylated PAMAM dendrimer [21] (Figure 1b).

The products were titrated to quantify the % carboxylic acid groups (using 1% bromothymol blue as indicator and NaOH as base), and characterized by FTIR and ^{1}H NMR.

Chemical conjugation of trypsin

The succinoylated β-cyclodextrin (103.0 mg) was dissolved in distilled water (2 mL). EDC (24.0 mg) was added and the reaction mixture was stirred for 10 min. Then, sulfo-NHS (29.4 mg) was added and the reaction mixture was stirred for a further 45 min. Trypsin (24.6 mg) was then added to the reaction mixture and the pH was adjusted to pH ~ 8 by addition of 0.5 mM NaOH solution dropwise. The reaction mixture was continued stirring for 18 h at room temperature. The conjugate was purified by FPLC, and the collected fractions were desalted using an Amicon Ultracel^{TM} tubing of molecular weight cut-off 10,000 g/mol before freeze-drying. The conjugate

was characterized by SDS-PAGE electrophoresis, GPC, and FPLC. Bradford protein assay was used to estimate the total protein content [22] with BSA standards.

Succinoylated PAMAM dendrimer (90 mg) was dissolved in distilled water (2 mL). EDC (14.3 mg) was added and the reaction mixture was stirred for 10 min. Then, sulfo-NHS (16.6 mg) was added and the reaction mixture was stirred for a further 45 min. Trypsin (26.8 mg) was then added to the reaction mixture (Figure 2). The remaining procedure, purification and characterization were performed as described for β-cyclodextrin-trypsin conjugates.

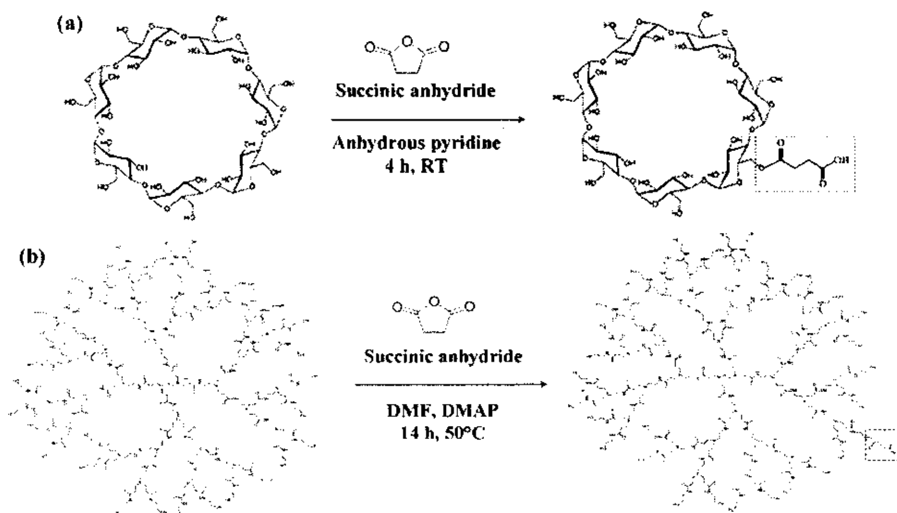

Figure 1 Reaction scheme of (a) succinoylated β-cyclodextrin and (b) succinoylated PAMAM

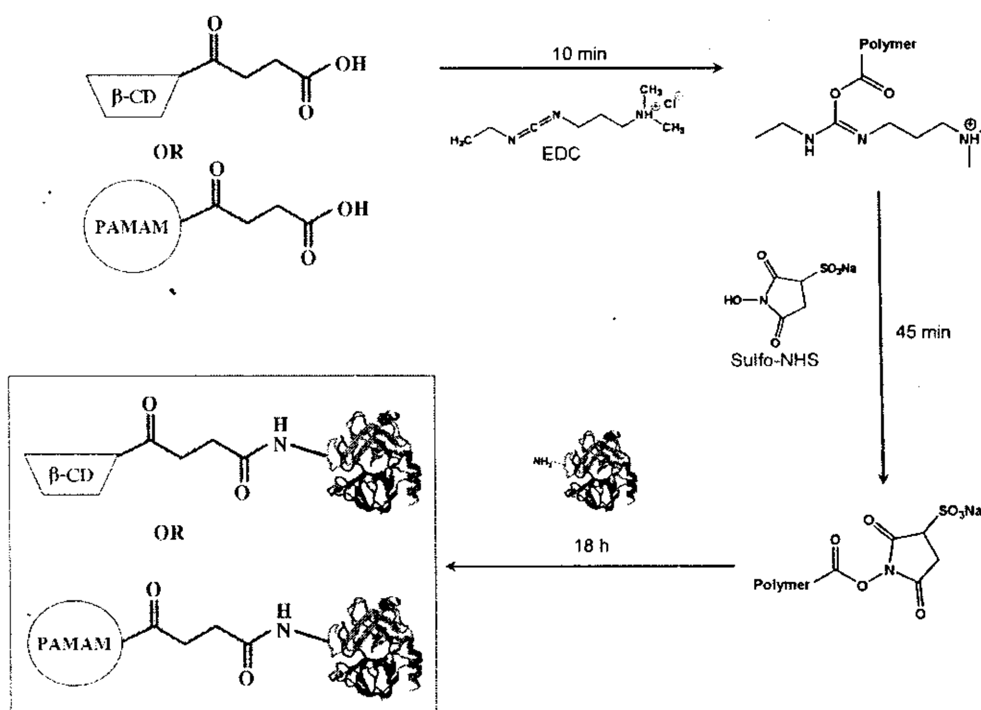

Figure 2 Reaction scheme for polymer-trypsin conjugates preparation

Enzyme activity determination

Activity of free and conjugated trypsin was determined using BAPNA as a substrate. A solution of each polymer-trypsin conjugates (equivalent to trypsin 2 μg in 0.1 M tris-HCl, pH 8.2 containing 20 mM $CaCl_2$) was added to the BAPNA solution (0.02-0.2 mg/ml in 0.1 M Tris-HCl, pH 8.2 containing 20 mM $CaCl_2$). The absorbance was measured at 410 nm after incubation at 30°C for 10 min.

Thermal stability and autolysis determination

Thermal stability was assessed as follows. Native trypsin and the polymer-trypsin conjugates were incubated in 0.1 M Tris-HCl, pH 8.2 containing 20 mM $CaCl_2$ for 10 min at temperature between 30-70°C [5]. Then the residual enzyme activity was assayed using BAPNA as described above.

To determine autolysis, native trypsin and polymer-trypsin conjugates were incubated at 40°C in 0.1 M Tris-HCl, pH 8.2 containing 20 mM $CaCl_2$ for 180 min. Aliquots (20 μL) of free trypsin or polymer-trypsin conjugates (2 μg protein-equiv.) were removed at different times and assayed for retained amidase activity using BAPNA as described above.

Results and discussion

Synthesis and characterization of the succinoylated polymer

Succinoylated β-CD and succinoylated PAMAM were successfully synthesized. FT-IR confirmed the presence of the ester functional group at 1731 and 1722 cm^{-1} in succinoylated β-CD (Figure 3a) and succinoylated PAMAM (Figure 3b), respectively. A degree of COOH content was assayed by titration. One molecule of succinoylated β-CD and succinoylated PAMAM contained 3 and 1.1 carboxylic groups, respectively. ^{1}H-NMR and ^{13}C-NMR confirmed the formation of succinoylated polymers. Succinoylated β-CD (Figure 4a) showed multiplet peaks of ethyl protons at 2.3-2.7 ppm which was not observed in spectrum of β-CD. ^{13}C-NMR spectrum of succinoylated PAMAM showed carbonyl of carboxylic and ester peak at 181.4 ppm and 176.3 ppm, respectively (Figure 4b).

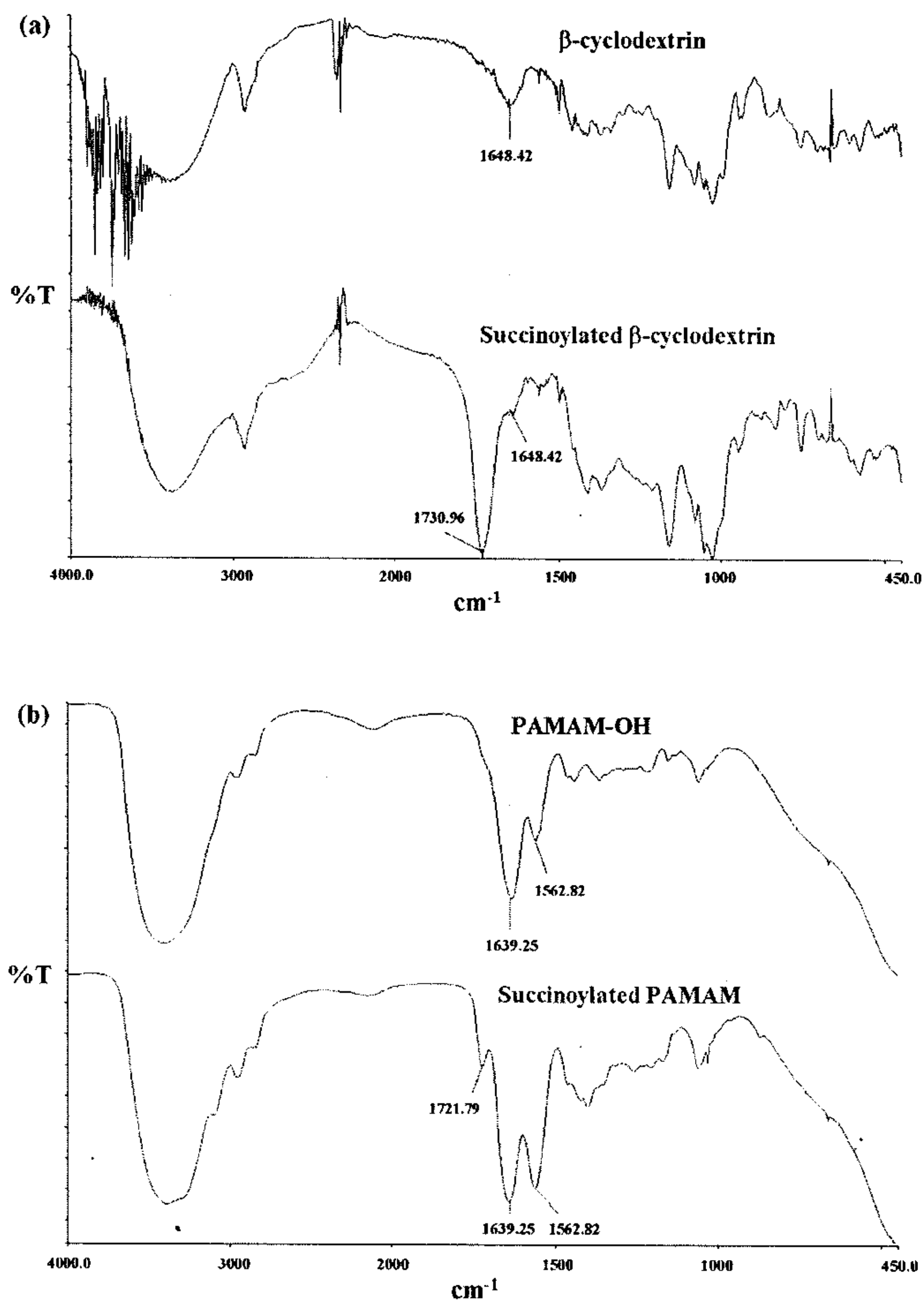

Figure 3 FT-IR characterization of (a) succinoylated β-CD and (b) succinoylated PAMAM compare to their parent polymers.

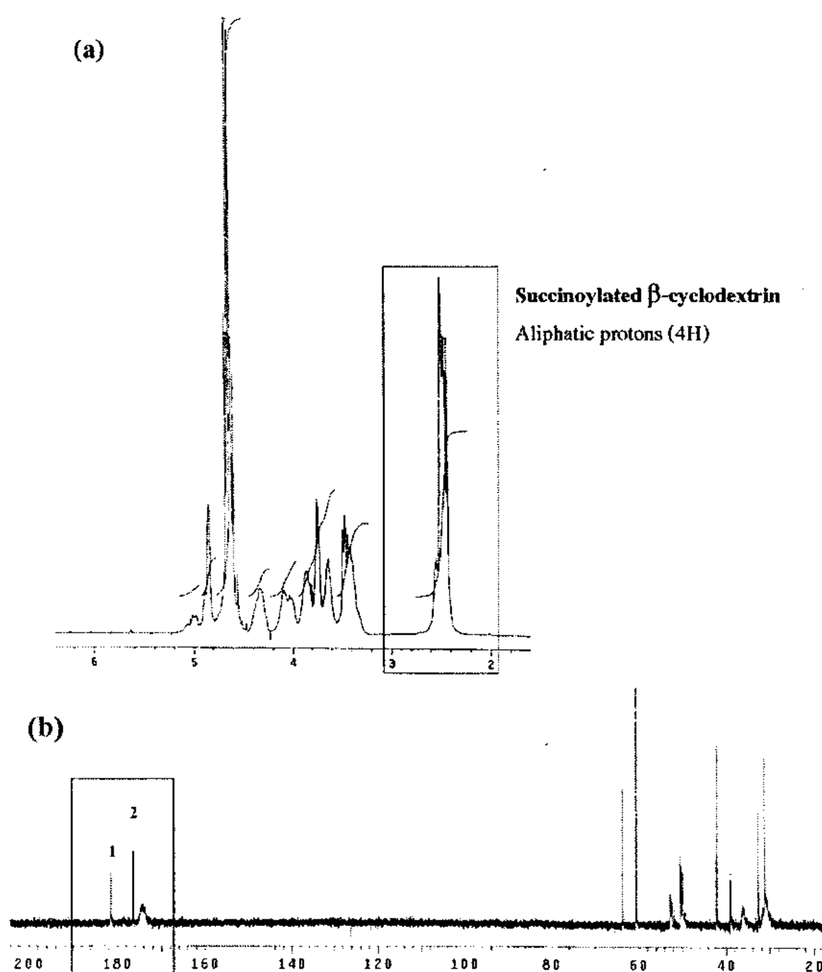

Figure 4 NMR spectra of succinoylated polymers. Panel (a) ^{1}H-NMR of succinoylated β-CD and Panel (b) ^{13}C-NMR of succinoylated PAMAM; peak (1) showed carbonyl of carboxylic at 181.4 ppm and peak (2) showed carbonyl of ester at 176.3 ppm.

Synthesis and characterization of the polymer-trypsin conjugates

β-CD - and PAMAM-trypsin conjugates were prepared and purified by FPLC to separate the conjugate from free trypsin (Figure 5a). The purity was confirmed by SDS-PAGE that no free trypsin was detected (Figure 5b).

The molecular weight of conjugate was predicted from FPLC standard curve as shown in Table I. The β-CD - and PAMAM-trypsin conjugates contained 72 and 50 wt% total protein and the ratio of polymer: trypsin was 6.7:1 and 2.6:1, respectively (Table I).

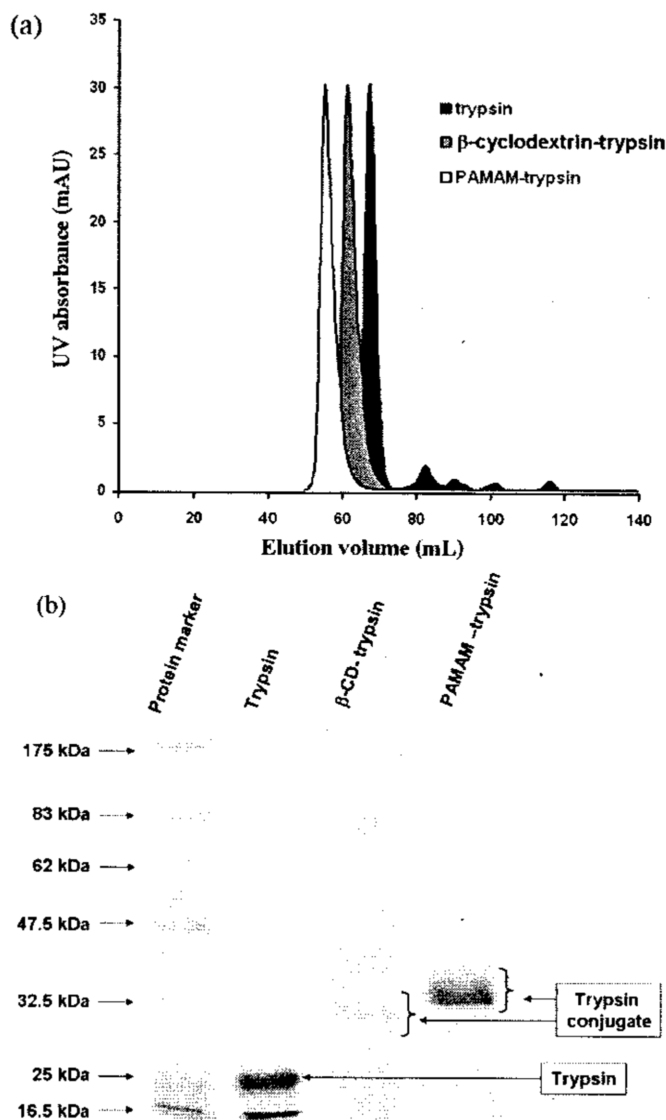

Figure 5 Characterization of β-CD- and PAMAM-trypsin conjugate. Panel (a) FPLC and Panel (b) SDS-PAGE

TABLE I CHARACTERISTIC OF POLYMER AND POLYMER-TRYPSIN CONJUGATES

	Protein content (wt%)	Mw (g/mol)	Polymer: trypsin ratio
Trypsin[a]	-	21,400[b]	-
β-CD	-	1,135	-
β-CD -trypsin	72	29,000[b]	6.7:1
PAMAM dendrimer	-	6,941	
PAMAM-trypsin	50	39,300[b]	2.6:1

[a]Trypsin from bovine pancreas (Mw 23,800; 223 amino acids) (http://www.sigmaaldrich.com)

[b]Estimated using protein FPLC standards (bovine pancreas ribonuclease A, Mw 13,700; chymotrypsinogen A, Mw 25,000; bovine pancreas ovalbumin, Mw 43,000 and hen egg albumin, Mw 67,000)

Enzymatic activity of trypsin and polymer-trypsin conjugates

The polymer-trypsin conjugates showed lower K_M, V_{max} and K_{cat}. The activity of β-CD and PAMAM-trypsin reduced to 13.9 and 29.2 %, respectively, compared to the free trypsin value (Table II). This result is similar to the previous studies which the reduction of trypsin activity was found after polymer conjugation [3, 23-26]. The enzymatic activity of trypsin also decreased after conjugation. It may be due to the denaturation of the protein structure during chemical modification, the modification of essential functional groups, refolding of the protein chain upon attachment of polymer, altering the active site making it less effective, or by steric hindrance preventing access of substrates to the active sites [27].

In this study, enzyme kinetic analysis using the Hanes-Woolfe plot indicated that Michaelis constant value (K_M) in Table II decreased after β-CD attachment compare to native trypsin. However, K_M value was similar to native trypsin upon attachment to PAMAM, suggesting that apparent affinity is not altered by polymer conjugation. It is likely to be due to steric hindrance restricting access of the substrate to the active site. Moreover, the conjugates displayed V_{max} values that were ~3-4 fold lower, and the substrate turnover rate K_{cat} was decreased ~3-7 fold lower (Table II).

TABLE II ENZYME ACTIVITY OF NATIVE AND POLYMER-TRYPSIN CONJUGATES

	Trypsin	β-CD-trypsin[a]	PAMAM-trypsin[a]
Activity (%)	100	13.9 ± 0.7	29.2 ± 1.8
K_M(mM)	0.29 ± 0.05	0.15 ± 0.04	0.26 ± 0.02
V_{max} (μM/min)	1.5 ± 0.19	0.37 ± 0.02	0.44 ± 0.03
K_{cat}(s^{-1})	0.30 ± 0.04	0.04 ± 0.01	0.09 ± 0.01

[a]Data shown relate to the trypsin control; mean ± SD (n=3)

Thermal stability and autolysis of trypsin and polymer-trypsin conjugates

The progressive decline in enzyme activity was detected for both polymer-trypsin conjugates and trypsin with incubation time at all temperatures (30-70°C) (Figure 6a, 6b and 6c). The β-CD -trypsin conjugate was more stable than native trypsin at all temperatures (Figure 7a). After incubation at different temperature (30-70°C) for 10 min, it has been found that the PAMAM-trypsin conjugate was inactivated at 50°C while β-CD -trypsin and native trypsin were inactivated at 70°C. However, thermal resistance were observed for trypsin, β-CD -trypsin and PAMAM-trypsin conjugates, expressed in the values of T_{50} of 47°C, 55.5°C and 44°C, respectively.

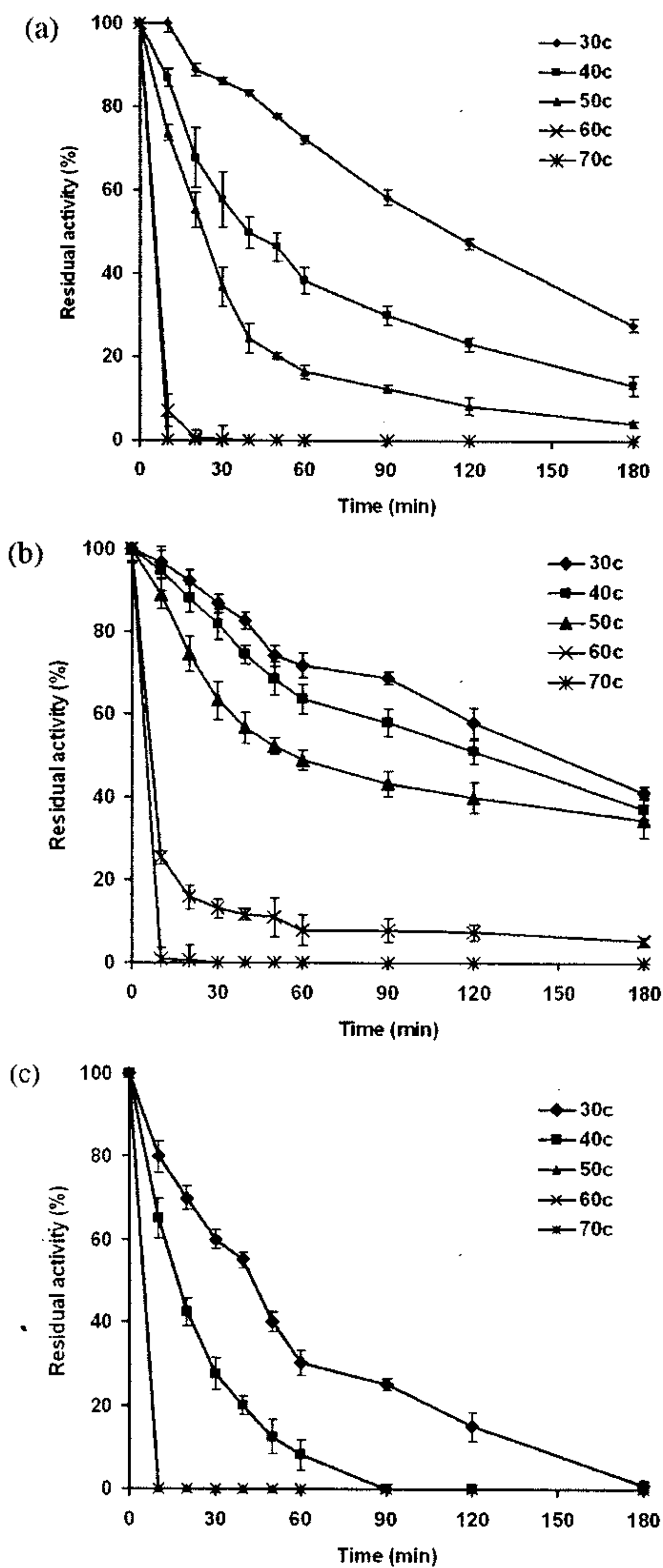

Figure 6 Effect of temperature (30-70°C) on the activity of (a) native bovine trypsin, (b) β-CD-trypsin conjugates and (c) PAMAM-trypsin conjugates measured using BAPNA as a substrate after incubation for 180 min (mean; n = 3).

Murphy and O'Fagain (1996) described the covalent modification of trypsin with acetic acid *N*-hydroxysuccinimide ethyl ester, and only an increase of 5°C was obtained for T_{50} [28]. Similarly, stabilization was also reported for trypsin modified with carboxymethylcellulose, obtaining showed increasing of 7°C for T_{50} under similar experimental conditions [25]. Several monosubstituted amino derivatives of β-CD were also used for modifying with carboxylic group of trypsin. Fernandez, et al. (2003) were performed the similar experiment, the results showed an increase of 12.5-14.5°C for T_{50} of β-CD -trypsin conjugates. Their results were similar to our studies which used the succinoylated β-CD containing carboxylic end group to coupling with amino group of trypsin. The spacer arm of polymer has influenced in chemical change in the protein structure and resulted in stability effect or efficacy of polymer-protein conjugates [5].

The β-CD-trypsin was more stable while the PAMAM-trypsin conjugate was more susceptible to autolysis at 40°C than native trypsin (Figure 7b). The half-life ($t_{1/2}$) calculated from the time course for autolysis was shown in Table III. With the same linker of conjugates, the order of $t_{1/2}$ was the β-CD -trypsin > PEG-trypsin conjugates > native bovine trypsin > PAMAM-trypsin conjugate.

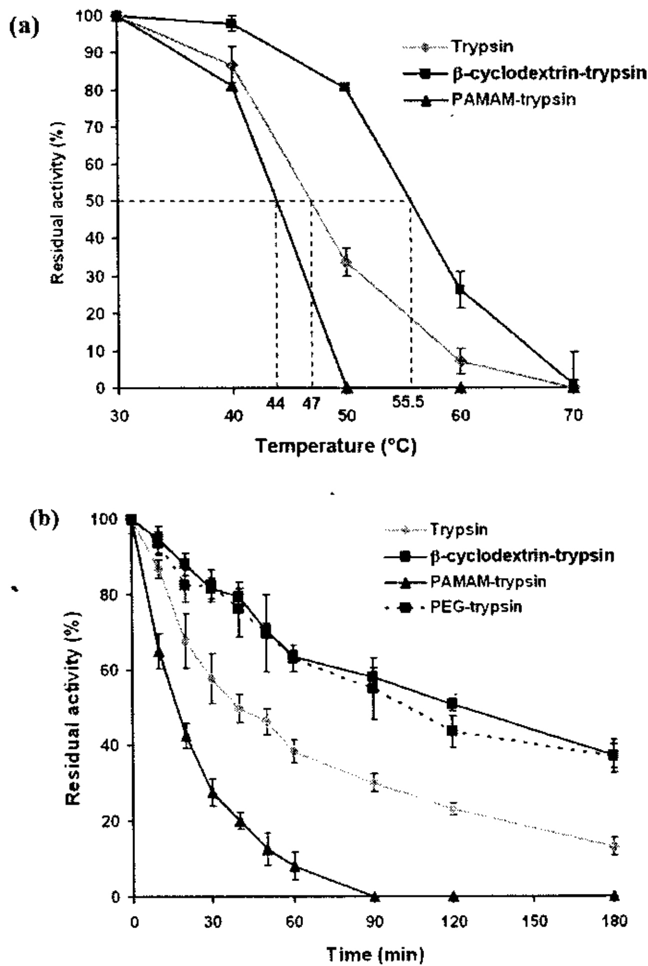

Figure 7 Autolysis (40 °C) of native, β-CD-, PAMAM- and PEG-trypsin conjugates measured using BAPNA as a substrate (mean; n = 3).

TABLE III HALF-LIFE ($T_{1/2}$) OF NATIVE AND POLYMER-TRYPSIN CONJUGATES

Compound	Half-life ($t_{1/2}$)[a]
Trypsin	40 ± 1
β-Cyclodextrin-trypsin conjugate	125 ± 3
PAMAM-trypsin conjugate	17 ± 1
PEG-trypsin conjugate[b]	102 ± 1

[a]Experiments were conducted at 40°C and the hydrolysis of BAPNA was used to measure residual trypsin activity. Data represent t1/2 min (mean ± SD) (n=3).

[b]Data taken from [3].

The possible structure of β-CD - and PAMAM-trypsin conjugates were shown in Figure 8. β-CD might be expected to enhance protection from autolytic attack due to steric hindrance by wrapping around protein [3, 27]. It could be due to the glycosidic –OH groups in poly(glucose) polymer can create hydrogen-bonding to protect and stabilize proteins [28].

Interestingly, the PAMAM-trypsin conjugates displayed lower thermal stability and had a lower $t_{1/2}$ for autolysis than other conjugates and native trypsin. Trypsin was less effective after conjugated with PAMAM dendrimer. It might be because of (i) a less steric hindrance of PAMAM structure due to a less number of PAMAM molecules in the conjugate, (ii) modifying at essential functional groups that maybe interfere the active site of trypsin [27], or (iii) the chemical modification of the trypsin with PAMAM dendrimer may cause some degree of aggregation [30].

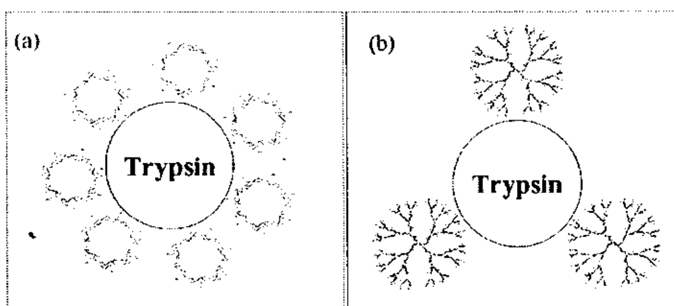

Figure 8 Possible structure of (a) β-CD-trypsin conjugates and (b) PAMAM-trypsin conjugates

Conclusion

The use of β-CD and PAMAM dendrimer as modifying agents for trypsin was described. The type and shape of polymer play important roles on kinetic parameters and enzyme activity. β-CD had a potential to use for protein conjugation. It can stabilize and protect protein from degradation better than PAMAM dendrimer. Modifying the protein drugs with this polymer is interesting area for further investigation.

Acknowledgements

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Appendix

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Pharmaceutical Nanotechnology

Effect of PEG molecular weight and linking chemistry on the biological activity and thermal stability of PEGylated trypsin

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Abstract

PEGylated proteins are routinely used as therapeutics, but systematic studies of the effect of PEG molecular weight and linking chemistry on the biological activity and particularly the thermal stability of the conjugated protein are rarely made. Here, activated monomethoxypolyethylene glycol (mPEG)s (Mw 1100, 2000 and 5000 g/mol) were prepared using succinic anhydride (SA), cyanuric chloride (CC) or tosyl chloride (TC) and used to synthesise a library of trypsin conjugates. The enzyme activity (K_M, V_{max} and K_{cat}) of native trypsin and the mPEG-modified trypsin conjugates was compared using *N*-benzoyl-L-arginine *p*-nitroanilide (BAPNA) as a substrate, and their thermal stability determined using both BAPNA and *N*-α-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) as substrates to measure amidase and esterase activity respectively. The effect of conjugate chemistry on trypsin autolysis was also examined at 40 °C. PEG-trypsin conjugates containing the higher molecular weight of mPEG (5000 g/mol) were more stable than free trypsin, and the conjugate containing CC-mPEG 5000 g/mol had the best thermal stability.
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Keywords: Monomethoxypolyethylene glycol (mPEG); PEG conjugates; Thermal stability; Trypsin

1. Introduction

The biotechnology revolution has produced many novel peptides and proteins that have become important new drugs. More than 80 are now marketed in the United States, and 350 are undergoing clinical trials (reviewed in Harris and Chess, 2003). However, protein drugs also possess several shortcomings that limit their usefulness. These include premature degradation due to susceptibility to destruction by proteolytic enzymes, short circulating half-life, low solubility, rapid kidney clearance of lower molecular weight proteins and potential immunogenicity. Conjugation of proteins and peptides to natural or synthetic polymers has shown the ability to solve many of these problems (reviewed in Harris and Chess, 2003; Pasut and Veronese, 2007).

Although a variety of polymers have been used for protein conjugation, polyethylene glycol (PEG) has been most popular due to its excellent water-solubility, and it is known to be safe being FDA approved for use in injectable, topical, rectal and nasal formulations. PEG is synthesised by anionic ring polymerisation of ethylene oxide using methanol or water as initiator to give a linear polyether that can be modified to ensure a single terminal reactive functional group e.g. monomethoxy PEG (mPEG). This enables protein modification without risk of cross-linking (reviewed in Pasut and Veronese, 2007; Duncan, 2003; Greenwald et al., 2003; Harris and Chess, 2003). Moreover, PEG polymerisation can be controlled to produce a wide range of discreet molecular weights (<5000–50,000 g/mol) with low polydispersity (Mw/Mn from <1.01 to 1.1 for higher PEGs of Mw ~50,000 g/mol). The high hydration and flexibility of the PEG chain enables a reduction in antigenicity of proteins to which it is bound, and careful choice of PEG molecular weight enables fine tuning of plasma pharmacokinetics

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and route of elimination (by renal and hepatic pathways) of specific peptides and proteins to suit their pharmacodynamic requirements.

Despite the large number of studies on PEG-protein modification, including the emergence of novel linking chemistry (Brocchini et al., 2006), there has been little attempt to systematically study the effect of PEG molecular weight and linking chemistry on protein properties such as thermal stability. Therefore, the aims of this study were to compare the enzyme activity (kinetic parameters), the thermal stability and autolysis of a library of PEG-modified trypsin conjugates synthesised using PEGs of different molecular weights (Mw 1100, 2000 and 5000 g/mol) and different linking chemistries. Trypsin (from bovine pancreas) was chosen as a convenient model protein. It is a pancreatic serine protease, composed of a single polypeptide chain of 223 amino acid residues, and it displays a narrow substrate specificity hydrolysing L-lysyl and L-argininyl bonds of polypeptides (amidase activity; Walsh, 1970 and esterase activity; Bergmeyer et al., 1974). As the covalent attachment of *p*-nitrophenyl chloroformate (NPC) activated mPEG to bovine pancreatic trypsin increased thermal stability (Gaertner and Puigserver, 1992; Zhang et al., 1999) the PEG-trypsin conjugates were synthesised using succinic anhydride (SA), cyanuric chloride (CC) or tosyl chloride (TC) to activate PEG (Scheme 1). The enzyme activities (K_M, V_{max} and K_{cat}) were determined by using *N*-benzoyl-L-arginine *p*-nitroanilide (BAPNA) and *N*-α-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) as substrates for amidase and esterase activity, respectively.

2. Materials and methods

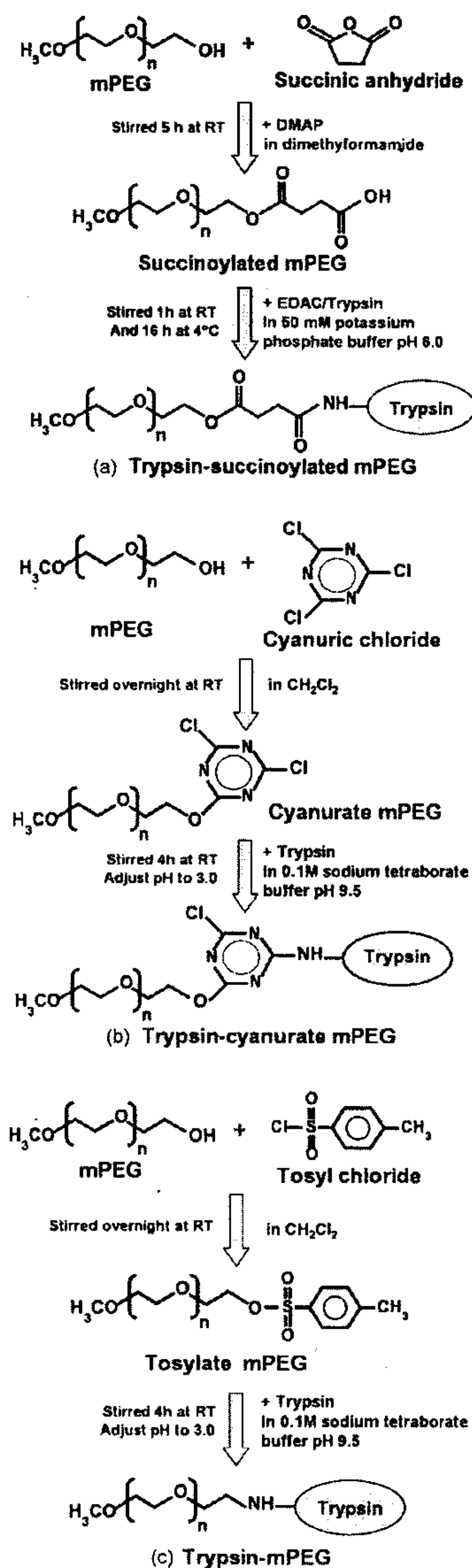
2.1. Materials

mPEGs of molecular weight 1100, 2000 and 5000 g/mol, succinic anhydride and 4-dimethylaminopyridine (DMAP) were from Fluka (Switzerland). Bovine pancreatic trypsin (EC 3.4.21.4), cyanuric chloride, tosyl chloride, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), 2,4,6-trinitrobenzene sulfonic acid (TNBS), BAPNA, BAEE, and the Coomassie blue for protein assay were from Sigma (Germany). All other reagents and chemicals were of analytical grade.

2.2. Methods

2.2.1. Preparation of activated mPEG

2.2.1.1. Succinoylated mPEG (SA-mPEG) (Scheme 1a). mPEG (1100, 2000 or 5000 g/mol; 1 mmol) was dried by azeotropic distillation using toluene and then dissolved in anhydrous dimethylformamide (DMF, 10 ml). Succinic anhydride (4 mmol) was added, followed by DMAP (4 mmol). The mixture was stirred overnight at room temperature, the resulting polymer was then precipitated using ether, recrystallized twice from CH_2Cl_2/ether (1:40) and characterised by TLC ($BuOH/AcOH/H_2O$, 4:1:1) (Zalipsky et al., 1983), FTIR and ^1H NMR. The acid groups were quantified by titration against stan-



Scheme 1. Reaction scheme for synthesis of (a) trypsin-succinoylated mPEG conjugates; (b) trypsin-cyanurate mPEG conjugates; (c) trypsin-mPEG conjugates.

dardized NaOH solution using bromothymol blue as an indicator (Hreczuk-Hirst et al., 2001).

2.2.1.2. Cyanurate mPEG (CC-mPEG) (Scheme 1b). Cyanuric chloride (3 mmol) was dissolved in CH_2Cl_2 containing anhydrous sodium sulphate. mPEG (1100, 2000 or 5000 g/mol; 1 mmol) was added and the mixture was stirred overnight at room temperature. The solution was then filtered, and ether was added slowly with stirring. The finely divided precipitate was collected on a filter and re-dissolved in CH_2Cl_2. This precipitation and filtration process was repeated several times until the ether was free of residual cyanuric chloride as determined by TLC ($CHCl_3$/MeOH, 7:1) following the method of Schiavon et al. (2004). The product was characterised by FTIR and ^{13}C NMR.

2.2.1.3. Tosylate mPEG (TC-mPEG) (Scheme 1c). Tosyl chloride (3 mmol) was dissolved in CH_2Cl_2 containing anhydrous sodium sulphate. mPEG (1100, 2000 or 5000 g/mol; 1 mmol) and triethylamine (1 mmol) was added and the mixture was stirred overnight at room temperature. The solution was filtered, and ether was added slowly with stirring. The resulting white powder was collected on a filter and re-dissolved in CH_2Cl_2, washed several times until the ether was free of residual tosyl chloride as determined by TLC ($CHCl_3$/MeOH, 9:1). The product was characterised by FTIR and ^1H NMR.

2.2.2. PEGylation of trypsin

Trypsin was modified with the activated mPEGs synthesised above as follows.

2.2.2.1. Trypsin-succinoylated mPEG (Trypsin-SA-mPEG) (Scheme 1a). EDAC was added to the reaction mixtures containing trypsin dissolved in 10 ml of 50 mM potassium phosphate buffer, pH 6.0, and succinoylated mPEGs of molecular weight 1000, 2000 and 5000 g/mol using a molar ratio of trypsin: succinoylated mPEGs (1:20, 1:40 and 1:60). The solution was stirred for 1 h at room temperature and then for 16 h at 4 °C. It was then dialysed at 4 °C for 48 h against water using a dialysis membrane of molecular weight cut-off 12,400 and the product was finally lyophilized (Fernandez et al., 2003).

2.2.2.2. Trypsin-cyanurate mPEG (Trypsin-CC-mPEG) (Scheme 1b). Cyanurate mPEG was reacted with trypsin solubilised in 0.1 M sodium tetraborate buffer (pH 9.5, 2 mg/ml). As above a molar ratio of trypsin: cyanurate mPEGs of 1:20, 1:40 and 1:60 was used. The resulting mixture was stirred at room temperature for 4 h, and then brought to pH 3.0, and dialysed at 4 °C as above and the product was lyophilised (Abuchowski and Davis, 1979).

2.2.2.3. Trypsin-tosylated mPEG (Tryp-TC-mPEG or Trypsin-mPEG) (Scheme 1c). Tosylated mPEG was reacted with trypsin solubilised in 0.1 M sodium tetraborate buffer (pH 9.5, 2 mg/ml) using the same molar ratios as given above. The resulting mixture was stirred at room temperature for 4 h, the solution then brought to pH 3.0, and dialysed at 4 °C as above and the product was lyophilised (Gaertner and Puigserver, 1992).

2.2.3. Characterisation of the trypsin conjugates

In all cases, the Bradford protein assay was used to determine the total protein content of the conjugates. The extent of the protein modification was also estimated by determining the number of remaining free amino groups using the TNBS assay as described by Fields (1971). Briefly, sample (20 μl) was added to 0.1 M sodium tetraborate buffer in 0.1 M NaOH (0.5 ml) and the volume was made up to 1 ml. Then, TNBS solution (20 μl) was added and the solution was rapidly mixed. After 5 min the reaction was stopped by adding 0.1 M sodium phosphate buffer containing 1.5 M sodium sulfite (2.0 ml), and the absorbance at 420 nm was determined. A blank was prepared similarly without sample.

2.2.4. Determination of trypsin (native and conjugate) activity using BAPNA

Amidase activity of native and the PEGylated-trypsin conjugates was measured using BAPNA as a substrate (Murphy and O'Fagain, 1996). Briefly, 4.2 mM BAPNA (18.2 mg dissolved in 1 ml DMSO and added to 9 ml of 0.1 M Tris–HCl, pH 8.2 containing 20 mM $CaCl_2$) (400 μl) and 0.1 M Tris–HCl, pH 8.2 containing 20 mM $CaCl_2$ (580 μl) were added to a 1 ml cuvette. Then, 20 μl of free trypsin or PEG-trypsin conjugate (10 μg protein-equiv. in 0.1 M Tris–HCl, pH 8.2 containing 20 mM $CaCl_2$) was added and the solution was thoroughly mixed. The mixture was then incubated in the water bath at 30 °C for 10 min. The absorbance was read using a Shimadzu UV 1601 Spectrometer (Kyoto, Japan) at 410 nm, and one unit of activity defined as the amount of enzyme that hydrolyses 1 μmol BAPNA/min. The trypsin activity was calculated using a range of substrate concentrations (0.4–2.0 mM) and a molar extinction coefficient 8800 M^{-1} cm^{-1} for free *p*-nitroaniline (PNA). The reaction volume was always 1.0 ml. To calculate the kinetic parameters, a Lineweaver–Burk plot (Erlanger et al., 1961) was constructed as follows:

$$\text{Unit of activity (unit/mg)} = (\Delta \text{Abs}_{410\,\text{nm}} \times 1000 \times 1.0)/(8800 \times \text{mg protein})$$

2.2.5. Determination of trypsin (native and conjugate) activity using BAEE

Esterase activity of native and PEGylated-trypsin conjugates was determined at 25 °C using BAEE as substrate. Briefly, 20 μl of free trypsin or PEG-trypsin conjugate (10 μg protein-equiv.) was added to a cuvette containing 3 ml of 0.25 mM BAEE in 67 mM sodium phosphate buffer, pH 7.6, then immediately mixed thoroughly by inversion. The increase in absorbance at 253 nm was measured for approximately 10 min and the trypsin activity was calculated for a range of substrate concentrations 0.025–0.25 mM. One unit of esterase activity was defined as the amount of enzyme that hydrolyses 1.0 mmol of BAEE/min at

25 °C (Bergmeyer et al., 1974) as follows:

$$\text{Unit of activity (unit/mg)} = [(\Delta \text{Abs}_{253\text{nm}}\ \text{test} - \Delta \text{Abs}_{253\text{nm}}\ \text{blank}) \times 1000 \times 3.02]/\text{mg protein}$$

2.2.6. Evaluation of the thermal stability of PEG-trypsin conjugates

To test the thermal stability, native trypsin and the PEGylated conjugates were incubated in aqueous buffer (1.67 mM of 0.1 M Tris–HCl, pH 8.2 containing 20 mM $CaCl_2$ for amidase activity and 0.25 mM of 67 mM sodium phosphate buffer, pH 7.6 for esterase activity) for 10 min at temperatures between 30 and 70 °C. Then the residual trypsin for amidase or esterase activity was assayed using BAPNA or BAEE as described above. The activities of samples were compared to that at 30 °C (Zhang et al., 1999; Fernandez et al., 2002, 2003).

2.3. Evaluation of autolysis of PEG-trypsin conjugates

Native trypsin and the PEGylated-trypsin conjugates were incubated at 40 °C in aqueous buffer (0.1 M Tris–HCl, pH 8.2 containing 20 mM $CaCl_2$ for amidase activity and 67 mM sodium phosphate buffer, pH 7.6 for esterase activity) for 180 min. Aliquots of free trypsin or PEG-trypsin conjugate (10 μg protein-equiv.) (20 μl) were taken at different times and assayed for amidase or esterase activity using BAPNA or BAEE as described above.

3. Results and discussion

3.1. Synthesis and characterisation of the activated mPEG intermediates and PEG-trypsin conjugates

The succinoylation of mPEGs of different molecular weight was monitored by TLC. No free succinic anhydride was detected in the product, and the content of acid groups was 87–98% by titration. FTIR of SA-mPEG showed disappearance of the characteristic of –OH signals of mPEG at 3461 cm^{-1}, with the characteristic ester (1731 cm^{-1}) and carboxyl group (–COOH) of succinoyl moiety (1647 cm^{-1}). ^{1}H NMR confirmed the succinoylated mPEG characteristic peaks through the aliphatic protons of succinoyl moiety at 2.5–2.7 ppm (4H) and multiplet band of PEG protons at 3.5–4.2 ppm. The FTIR spectra of CC-mPEG showed disappearance characteristic of –OH moiety of mPEG at 3470 cm^{-1}, with a C=N characteristic peak at 1700 cm^{-1} and the skeleton vibration of the 1,3,5-triazine ring at 803 and 1500 cm^{-1}. ^{13}C NMR showed 1,3,5-triazine carbon ring at 172.1–174.2 ppm and carbon skeleton of PEG at 69.7–72.7 ppm. Similarly for the TC-mPEG the FTIR spectra showed disappearance characteristic of –OH moiety of mPEG at 3413 cm^{-1} with, in this case, the characteristic SO_2 (1700 cm^{-1}) and the aromatic (at 1398 and 1176 cm^{-1}) peaks. ^{1}H NMR confirmed aromatic protons at 7.4–7.8 ppm and multiplet band of PEG protons at 3.2–3.7 ppm (results not shown).

Table 1
Estimated number of amino groups substituted in trypsin

Conjugate	Molar ratios of trypsin and PEG	mPEG 1100[a]	mPEG 2000[a]	mPEG 5000[a]
Tryp-SA-mPEG	1:20	4.4 ± 0.7	4.6 ± 0.1	5.4 ± 0.1
Tryp-SA-mPEG	1:40	5.3 ± 1.2	3.2 ± 0.2	4.8 ± 0.1
Tryp-SA-mPEG	1:60	5.2 ± 0.8	2.6 ± 0.2	5.2 ± 0.1
Tryp-CC-mPEG	1:20	8.5 ± 0.4	6.9 ± 0.1	7.6 ± 0.2
Tryp-CC-mPEG	1:40	8.6 ± 0.1	5.2 ± 0.5	6.8 ± 0.1
Tryp-CC-mPEG	1:60	8.5 ± 0.3	4.0 ± 0.3	4.8 ± 0.8
Tryp-TC-mPEG	1:20	9.1 ± 0.1	9.7 ± 0.1	8.5 ± 0.4
Tryp-TC-mPEG	1:40	8.9 ± 0.1	9.5 ± 0.1	7.0 ± 0.2
Tryp-TC-mPEG	1:60	9.0 ± 0.1	8.9 ± 0.1	7.0 ± 0.8

It should be noted that native trypsin has ~11 amino groups in the TNBS assay.
[a] Data show mean ± S.D. ($n = 3$).

These activated mPEG intermediates were then used to prepare the library of trypsin conjugates. All the molecular weight PEGs produced comparable levels of amino groups substitution of trypsin (Table 1). When experiments were carried out to ascertain the optimum ratio between trypsin:activated mPEG (reactions at molar ratios of 1:20, 1:40 and 1:60), a reaction ratio of 1:20 gave an adequate degree of substitution; increased amounts of the activated mPEG concentration did not show a higher degree of substitution. The TNBS assay indicated that native trypsin had 11 accessible amino groups compared to the primary structure trypsin from bovine pancreas which contains 14 lysine amino groups (Walsh, 1970). After modification, it appeared that 4–9 mPEG molecules were covalently bound depending on the reaction conditions and specific mPEG used. For all PEG molecular weights, the degree of modification was achieved using TC-mPEG > CC-mPEG > SA-mPEG (Table 1). This is probably due to the fact that tosyl chloride is a better leaving group for the conjugation condition used.

3.2. Enzyme activity of trypsin and PEG-trypsin conjugates measured using BAPNA

The kinetic parameters obtained for both native trypsin and the PEGylated-trypsin conjugates (measured using BAPNA as substrate) are summarised in Table 2. All conjugates had lower activity than native trypsin. The conjugates synthesised using higher molecular weight PEGs displayed much lower K_M values indicating a higher affinity for the binding site of trypsin. Increasing molecular weight of mPEGs resulted in a decrease in the K_M and V_{max} values seen. These results were in contrast to the observation of Gaertner and Puigserver (1992) as they found that all conjugates had a higher amidase activity (in terms of percentage) than trypsin. In addition, the influence of the linkers on the enzyme efficiency of the conjugates as reported in Table 2 for K_{cat}/K_M values should be noted. Modification of trypsin with cyanurate mPEG increased this catalytic parameter more than seen for the other conjugates. The trypsin conjugate prepared using succinoylated mPEG displayed a higher rate of hydrolysis (mM/min) than other conjugates at 30 °C (Fig. 1). This was likely due to the fact that it contained fewer mPEG chains than the other conjugates resulting in lower steric hindrance of the trypsin active site (Veronese, 2001).

Table 2
Enzyme activity

Conjugate	Residual activity (%)	K_M (mM)	V_{max} (min)	K_{cat} (s^{-1})	K_{cat}/K_M (s^{-1} mM^{-1})
Trypsin	100	0.88 ± 0.09	0.177 ± 0.002	6.1 ± 0.8	6.9 ± 0.2
Tryp-SA-mPEG 1100	96.2 ± 2.0	1.15 ± 0.16	1.047 ± 0.389	6.6 ± 0.6	6.4 ± 0.6
Tryp-SA-mPEG 2000	76.8 ± 9.5	0.93 ± 0.09	0.457 ± 0.061	5.3 ± 0.8	5.1 ± 0.8
Tryp-SA-mPEG 5000	65.2 ± 8.3	0.79 ± 0.03	0.206 ± 0.026	4.0 ± 0.5	4.8 ± 0.9
Tryp-CC-mPEG 1100	17.5 ± 0.9	0.83 ± 0.09	0.104 ± 0.025	1.1 ± 0.2	1.4 ± 0.4
Tryp-CC-mPEG 2000	49.0 ± 9.8	0.45 ± 0.13	0.089 ± 0.017	2.7 ± 0.6	6.0 ± 1.0
Tryp-CC-mPEG 5000	92.9 ± 8.7	0.34 ± 0.07	0.040 ± 0.004	4.7 ± 0.5	14.4 ± 2.3
Tryp-TC mPEG 1100	19.9 ± 2.4	0.71 ± 0.11	0.160 ± 0.014	1.2 ± 0.1	1.7 ± 0.3
Tryp-TC mPEG 2000	68.5 ± 0.3	0.66 ± 0.02	0.063 ± 0.001	4.1 ± 0.1	6.1 ± 0.2
Tryp-TC mPEG 5000	67.6 ± 0.6	0.48 ± 0.06	0.036 ± 0.010	3.4 ± 0.5	7.1 ± 0.2

Kinetic parameters of native and PEG-trypsin conjugates. In all cases the conjugates were prepared using a molar ratio of 1:20 (mean ± S.D.; $n = 3$).

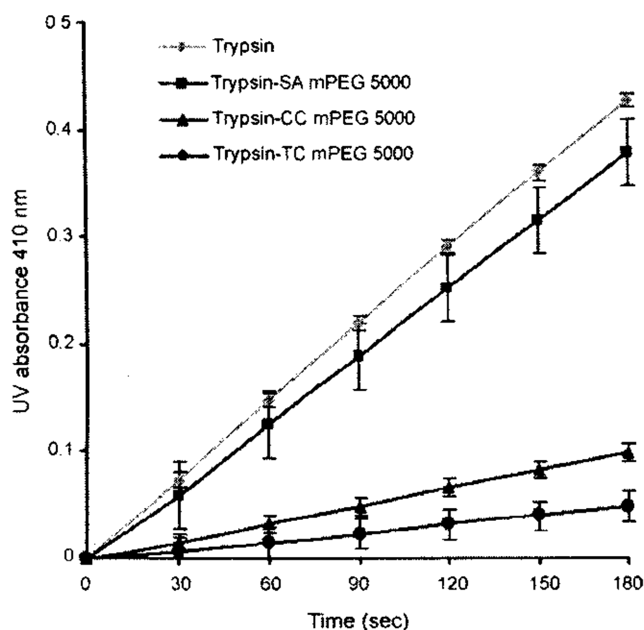


Fig. 1. Comparison of the activity of native and PEG-modified trypsin conjugates. The PEG used had a molecular weight of 5000 g/mol and in each case 10 μg/ml protein (trypsin) equivalent was used (mean ± S.D.; $n = 3$).

3.3. Thermal stability and autolysis of trypsin and PEG-trypsin conjugates

All mPEG-modified trypsins showed increased thermal stability compared to native trypsin (Figs. 2 and 3). This was true at all temperatures studied (except 70 °C) and when both BAPNA (Fig. 2) and BAEE (Fig. 3) were used as substrates. The highest activities were found when using the PEG with molecular weight of 5000 g/mol in all cases. This may be due to greater protection given by the higher molecular weight polymer chain; the hydrophilic PEG chain can swell and wrap around trypsin. Moreover, the PEG chain can form a highly hydrogen-bonded structure around the trypsin molecule (Gaertner and Puigserver, 1992; Zhang et al., 1999).

The time course for autolysis for native and modified trypsin at 40 °C was determined using both BAPNA and BAEE as substrates (Figs. 4 and 5 respectively). The half-life ($t_{1/2}$) of the PEG-trypsin conjugates was increased up to 4 fold compared to native trypsin (Table 3), and trypsin-modified with mPEG 5000 displayed the highest percentage retention of residual activity using both substrates and for all types of linker used. The values of residual activity measured using BAPNA as substrate were: native trypsin (13%), trypsin-SA-mPEG 5000 (37%), trypsin-CC-mPEG 5000 (50%) and trypsin-TC mPEG 5000 (39%) respectively. Stability was thus in the order CC > TC > SA, and moreover the same result was observed when using BAEE as substrate (residual activity 2, 8, 75 and 38% respectively).

All the mPEG-trypsin conjugates were less prone to autolysis compare to the native trypsin and they showed similar autolysis patterns. As the molecular weight of mPEG decreased the conjugates were less stable. This increased stability might be explained by specific modification of lysine residues (remembering the substrate specificity of trypsin) as

Table 3
Half-life of native and PEG-trypsin conjugates

Conjugate	Substrate[a]							
	BAPNA				BAEE			
	Trypsin	mPEG 1100	mPEG 2000	mPEG 5000	Trypsin	mPEG 1100	mPEG 2000	mPEG 5000
Trypsin	41 ± 1	–	–	–	8 ± 1	–	–	–
Tryp-SA-mPEG	–	45 ± 1	56 ± 1	102 ± 1	–	8 ± 1	9 ± 1	13 ± 1
Tryp-CC-mPEG	–	73 ± 1	119 ± 2	180 ± 2	–	15 ± 1	20 ± 1	>180
Tryp-TC-mPEG	–	95 ± 2	127 ± 3	157 ± 3	–	12 ± 1	15 ± 1	60 ± 1

Experiments were conducted at 40 °C and the hydrolysis of BAPNA and BAEE was used to measure residual trypsin activity.
[a] Data represent $t_{1/2}$ min (mean ± S.D.) ($n = 3$).

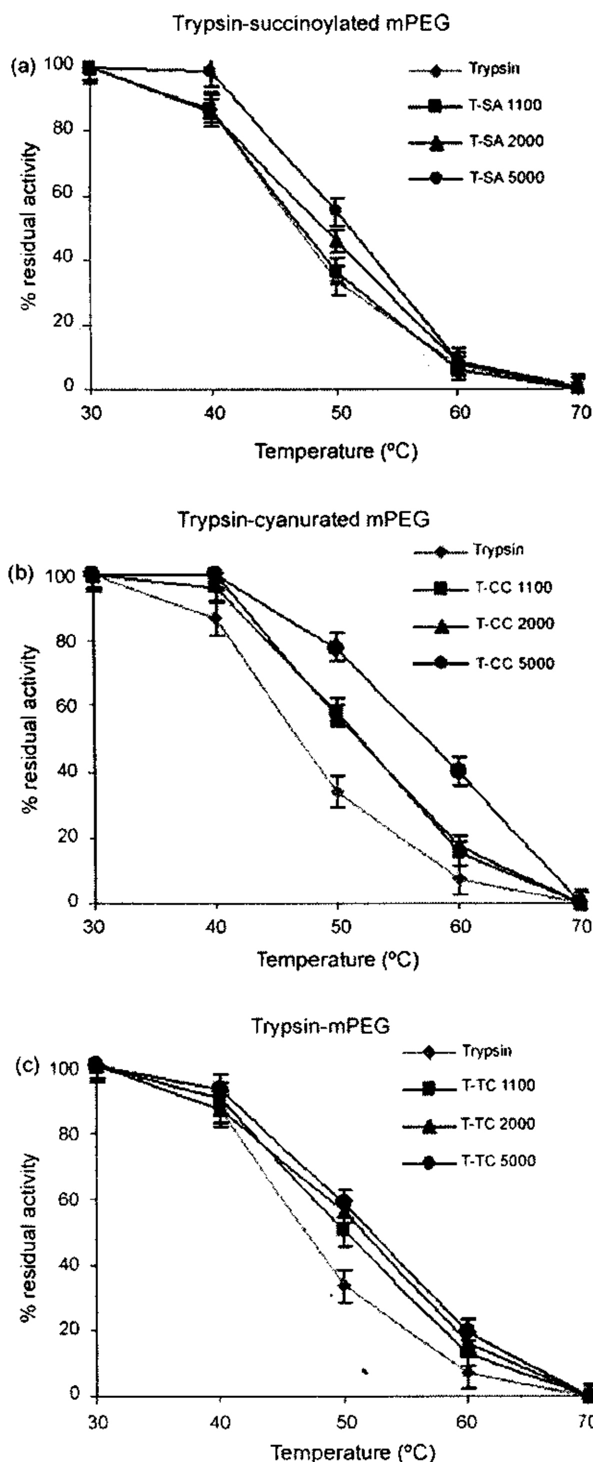


Fig. 2. Effect of temperature on the activity of native and PEG-modified trypsin conjugates measured using BAPNA as a substrate (mean ± S.D.; $n = 3$).

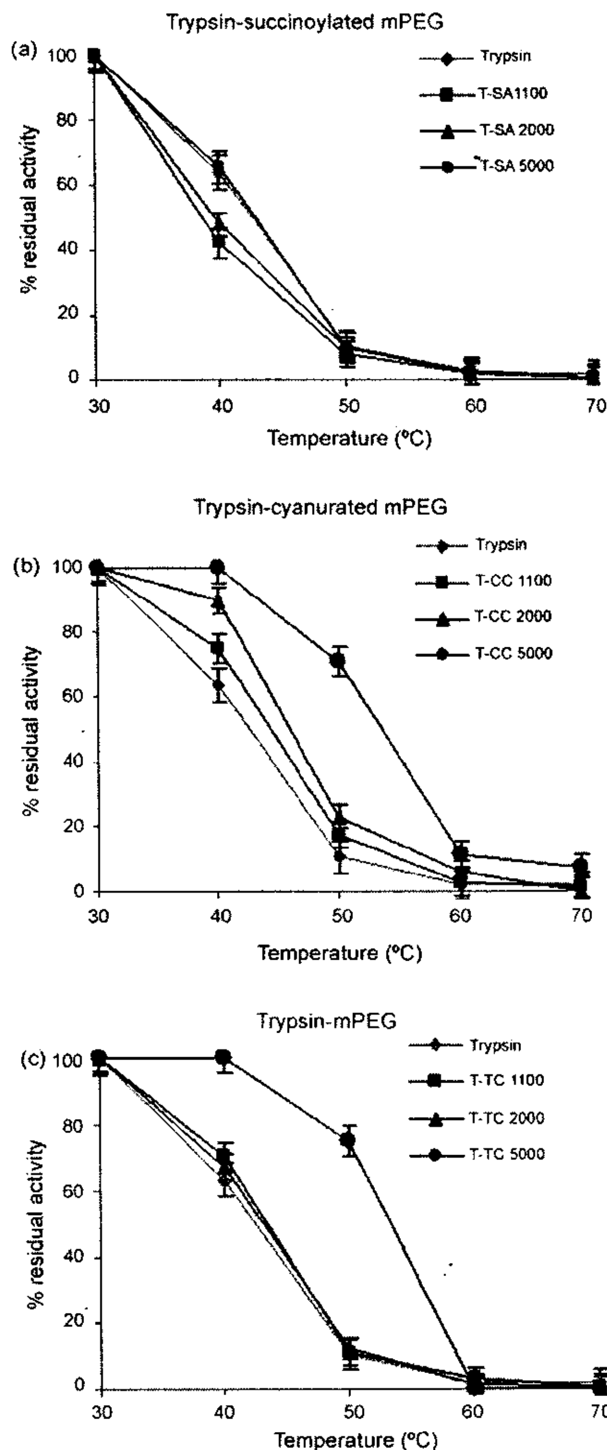


Fig. 3. Effect of temperature on the activity of native and PEG-modified trypsin conjugates measured using BAEE as a substrate (mean ± S.D.; $n = 3$).

this would inherently be expected to protect against autolysis, and/or the fact that the mPEG chains attached on the enzyme surface would anyhow cause non-specific steric hindrance (Murphy and O'Fagain, 1996). However, the observation that the higher molecular weight PEGs produced greater trypsin stability suggest a major role for the latter mechanism. The increasing of thermal stability of modified trypsins could also result from the formation of a highly hydrogen-bonded structure, with polymer wrapped around the protein (Gaertner and Puigserver, 1992; Zhang et al., 1999).

In the context of therapeutic protein formulation development, the active ingredient must be stable during formulation manufacture and for the shelf-life of the product. As thermal stability can be a good predictor of activity it is important to note that trypsin-modified with mPEG 5000 showed better stability

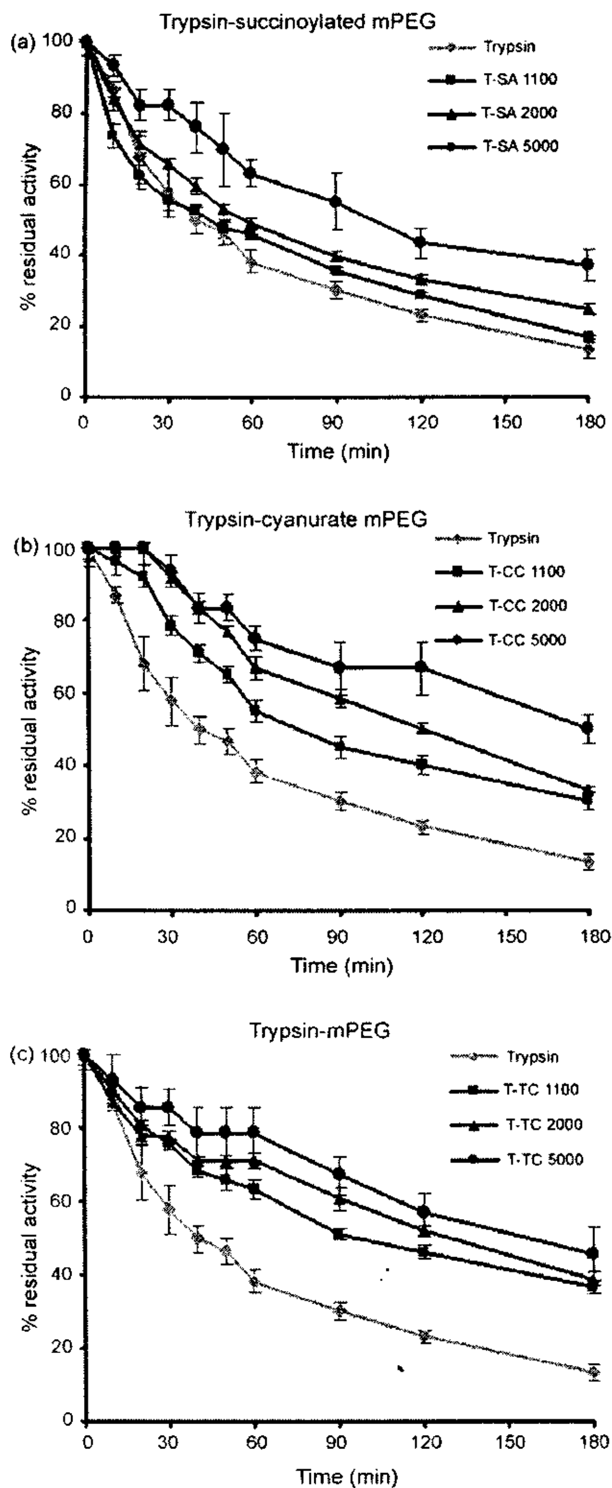


Fig. 4. Autolysis of native and PEG-modified trypsin conjugates measured using BAPNA as a substrate (mean ± S.D.; $n = 3$).

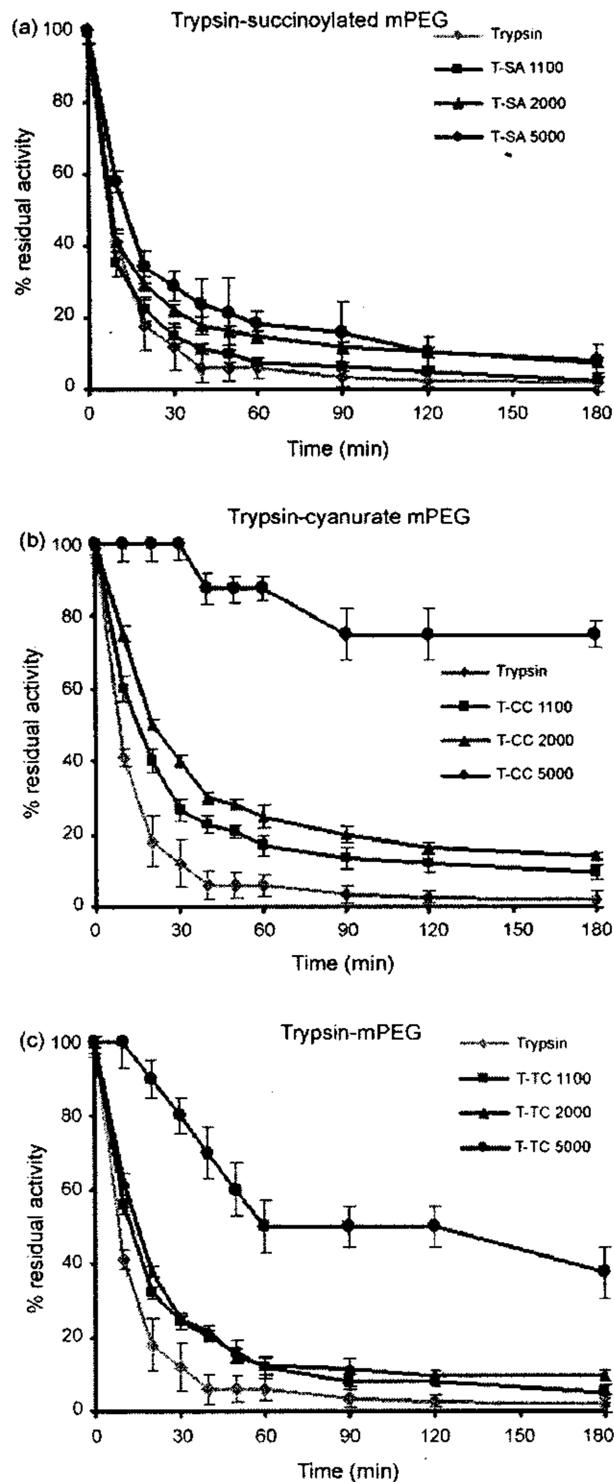


Fig. 5. Autolysis of native and PEG-modified trypsin conjugates measured using BAEE as a substrate (mean ± S.D.; $n = 3$).

compared to the native trypsin when tested at high temperature (Fig. 2). This study therefore underlines that, not only is the choice of PEG molecular weight and linking chemistry important in terms of retained biological activity of the protein, it may have an important impact on issues relating to formulation development.

4. Conclusions

In this study, trypsin was successfully conjugated with mPEG of molecular weight 1100, 2000 and 5000 g/mol. Although chemical modification of mPEG to trypsin could decrease activity of trypsin, but the modified trypsin conjugates containing the higher molecular mass mPEG showed higher affinity to bind-

ing site of enzyme with substrate, higher thermal stability, more stable against autolysis and had an increased $t_{1/2}$ compared to the native enzyme. The trypsin-conjugated mPEG 5000 having cyanurate linker demonstrated the best thermal stability.

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Chemical modification and thermal stability study of β-cyclodextrin- and PAMAM-trypsin conjugates

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Abstract — **Bovine pancreatic trypsin conjugates were first synthesized using succinoylated β-cyclodextrin and succinoylated PAMAM dendrimer. Their conjugates had a trypsin content of 72 and 50 wt %, respectively. The trypsin activity of conjugates measured by using *Nα*-benzoyl-*D*, *L*-arginine-*p*-nitroanilide hydrochloride (BAPNA) as substrate was decreased by polymer conjugation. The kinetic parameters (K_M, V_{max} and K_{cat}) were also calculated. The β-cyclodextrin-trypsin conjugate was more stable than native trypsin and PAMAM-trypsin conjugate at all temperatures between 30-70°C. It also exhibited better thermal stability in the autolysis assays at 40°C.**

Keywords— β-cyclodextrin, PAMAM dendrimer, polymer-protein conjugates, thermal stability

I. INTRODUCTION

The use of enzymes as therapeutic agents is often limited by the low resistance to proteolytic enzyme, short circulating half-life and immunogenicity of these proteins [1]. During the last few decades, several efforts have been devoted to the development of new methodologies for increasing functional stability of protein without affecting their biological behavior. Polymer conjugation, especially using PEG has been broadly used to reduce their immunogenicity and improve both the resistance to proteolytic degradation and thermal stability [2]. Our recent studies, a series of PEG-trypsin conjugates were synthesized using PEGs of different molecular weights and with different linking chemistries. The effect of chemical compositions on bioactivities of the conjugated enzyme, its autolysis and its thermal stability were studied. It was found that both molecular weight of PEG and the linkers play important roles on their activity and stability [3].

Historically, carbohydrate compounds have been extensively used as modifying agents for enzymes. β-cyclodextrin (β-CD, 1,135 g/mol) is a cyclic non-reducing α-(1-4)-linked D-glucopyranose composed of 7 glucose units in the chair conformation. The structure of these remarkable molecular receptors resembles a truncated annular cone with a central cavity, which is hydrophobic in nature and has the appropriate size to include a wide variety of hydrophobic guest compounds [4]. Previous study reported that amino derivatives of β-cyclodextrin can be coupled to aspartic and glutamic acid residues of proteins under mild conditions [5]. β-cyclodextrin was conjugated to many enzymes such as trypsin [5-7], α-chymotrypsin [8] and oxytocin [9]. Therefore, β-cyclodextrin was chosen in this study and modified with a different linker using succinoylation before conjugation to amino group of trypsin.

Polyamidoamine (PAMAM) dendrimer is another interesting class of drug carriers. It has well-define structure and consists of a central core and branched monomers. It has a globular shape and a large number of end groups. The ideal dendrimer should exhibit high aqueous solubility and drug-loading capacity, biodegradability, low toxicity, favorable retention and biodistribution characteristics, specificity, and appropriate bioavailability [10]. PAMAM dendrimer can be classified by the surface or terminal groups such as amine (-NH_2), carboxyl (-COOH) and hydroxyl (-OH) groups [11]. PAMAM-NH_2 and PAMAM-OH were widely used for conjugation to many drugs such as 5-ASA [12], ibuprofen [13], methotrexate [14], methylprednisolone [15], paclitaxel [16], Penicillin V [17], propanolol [18]. However, the use of this polymer for conjugation to enzymes has not been reported. PAMAM-OH, G3 (6,941 g/mol) was chosen to bound with trypsin in this study because: PAMAM-OH has many hydroxyl group similar to β-cyclodextrin.

Trypsin is widely used as a model protein. It is a well-studied serine protease involved in the digestion process of mammals that cleaves specific peptide bonds having a lysine or arginine residue [19].

Therefore, the aim of this study was to examine the effect of β-cyclodextrin and PAMAM dendrimer on activity, autolysis and thermal stability of trypsin in conjugation system.

II. MATERIALS

β-cyclodextrin was purchased from Fluka (USA) and PAMAM dendrimer (-OH) was from Aldrich (USA). *N*-Hydroxysulfosuccinimide sodium salt (sulfo-NHS) was

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purchased from Fluka (Switzerland). Bovine serum albumin (BSA) was from Sigma (Switzerland). Trypsin from bovine pancreatic, *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) and *Nα*-benzoyl-*D, L*-arginine-*p*-nitroanilide hydrochloride (BAPNA) were supplied by Sigma (UK). All chemicals used in this study were analytical grade.

III. METHODS

A. Succinoylation of polymers

β-cyclodextrin (0.5 g, 0.38 mmol) and succinic anhydride (0.5 g, 5 mmol) were dissolved in anhydrous pyridine (10 ml) for 4 h. The succinoylated β-cyclodextrin was then precipitated by addition of 40 ml isopropyl alcohol. The precipitate was then washed 3 times with 10 ml isopropyl alcohol [20] (Figure 1a).

PAMAM dendrimer (1 mmol) was dissolved in DMF (5 ml) and succinic anhydride (32 mmol) was added followed by DMAP (32 mmol). The reaction mixture was purged with N_2, sealed and stirred overnight at 50°C, under N_2. It was then poured into vigorously stirred diethyl ether (1 L) and stirred for 10 h. The ether was removed by filtration under vacuum and the remaining solid was dissolved in minimum amount of distilled water, poured into a dialysis membrane (molecular weight cut-off 3,500 g/mol) and dialyzed against distilled water for 48 h. The resulting solution was freeze-dried to yield succinoylated PAMAM dendrimer [21] (Figure 1b).

The products were titrated to quantify the % carboxylic acid groups (using 1% bromothymol blue as indicator and NaOH as base), and characterized by FTIR and ^{1}H NMR.

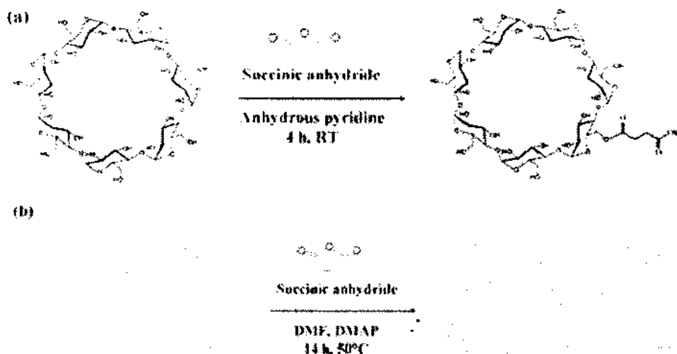

Figure 1 Reaction scheme of (a) succinoylated β-cyclodextrin and (b) succinoylated PAMAM

B. Chemical conjugation of trypsin

The succinoylated β-cyclodextrin (103.0 mg) was dissolved in distilled water (2 mL). EDC (24.0 mg) was added and the reaction mixture was stirred for 10 min. Then, sulfo-NHS (29.4 mg) was added and the reaction mixture was stirred for a further 45 min. Trypsin (24.6 mg) was then added to the reaction mixture and the pH was adjusted to pH ~ 8 by addition of 0.5 mM NaOH solution dropwise. The reaction mixture was continued stirring for 18 h at room temperature. The conjugate was purified by FPLC, and the collected fractions were desalted using an Amicon Ultracel™ tubing of molecular weight cut-off 10,000 g/mol before freeze-drying. The conjugate was characterized by SDS-PAGE electrophoresis, GPC, and FPLC. Bradford protein assay was used to estimate the total protein content [22] with BSA standards.

Succinoylated PAMAM dendrimer (90 mg) was dissolved in distilled water (2 mL). EDC (14.3 mg) was added and the reaction mixture was stirred for 10 min. Then, sulfo-NHS (16.6 mg) was added and the reaction mixture was stirred for a further 45 min. Trypsin (26.8 mg) was then added to the reaction mixture (Figure 2). The remaining procedure, purification and characterization were performed as described for β-cyclodextrin-trypsin conjugates.

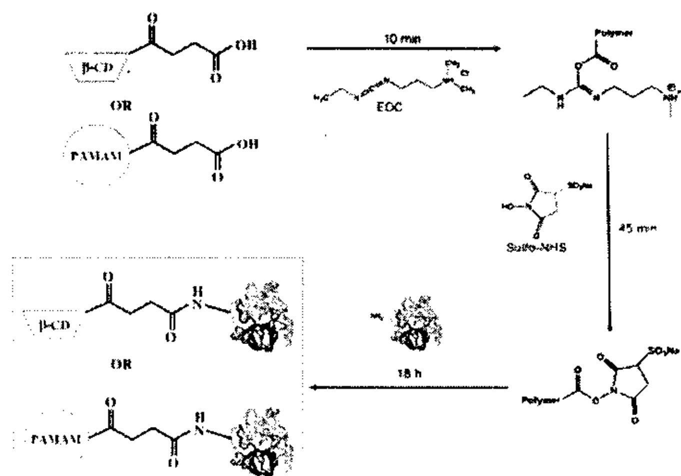

Figure 2 Reaction scheme for polymer-trypsin conjugates preparation

C. Enzyme activity determination

Activity of free and conjugated trypsin was determined using BAPNA as a substrate. A solution of each polymer-trypsin conjugates (equivalent to trypsin 2 μg in 0.1 M tris-HCl, pH 8.2 containing 20 mM $CaCl_2$) was added to the BAPNA solution (0.02-0.2 mg/ml in 0.1 M Tris-HCl, pH 8.2 containing 20 mM $CaCl_2$). The absorbance was measured at 410 nm after incubation at 30°C for 10 min.

D. Thermal stability and autolysis determination

Thermal stability was assessed as follows. Native trypsin and the polymer-trypsin conjugates were incubated in 0.1 M Tris-HCl, pH 8.2 containing 20 mM $CaCl_2$ for 10 min at temperature between 30-70°C [5]. Then the residual enzyme activity was assayed using BAPNA as described above.

To determine autolysis, native trypsin and polymer-trypsin conjugates were incubated at 40°C in 0.1 M Tris-HCl, pH 8.2 containing 20 mM $CaCl_2$ for 180 min. Aliquots (20 μL) of free trypsin or polymer-trypsin conjugates (2 μg protein-equiv.) were removed at different times and assayed for retained amidase activity using BAPNA as described above.

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IV. Results and Discussion

A. *Synthesis and characterization of the succinoylated polymer*

Succinoylated β-cyclodextrin and succinoylated PAMAM were successfully synthesized. FT-IR confirmed the presence of the ester functional group at 1731 and 1722 cm^{-1} in succinoylated β-cyclodextrin (Figure 3a) and succinoylated PAMAM (Figure 3b), respectively.

A degree of COOH content was assayed by titration. One molecule of succinoylated β-cyclodextrin and succinoylated PAMAM contained 3 and 1.1 carboxylic groups, respectively.

^{1}H-NMR and ^{13}C-NMR confirmed the formation of succinoylated polymers. Succinoylated β-cyclodextrin (Figure 4a) showed multiplet peaks of ethyl protons at 2.3-2.7 ppm which was not observed in spectrum of β-cyclodextrin. ^{13}C-NMR spectrum of succinoylated PAMAM showed carbonyl of carboxylic and ester peak at 181.4 ppm and 176.3 ppm, respectively (Figure 4b).

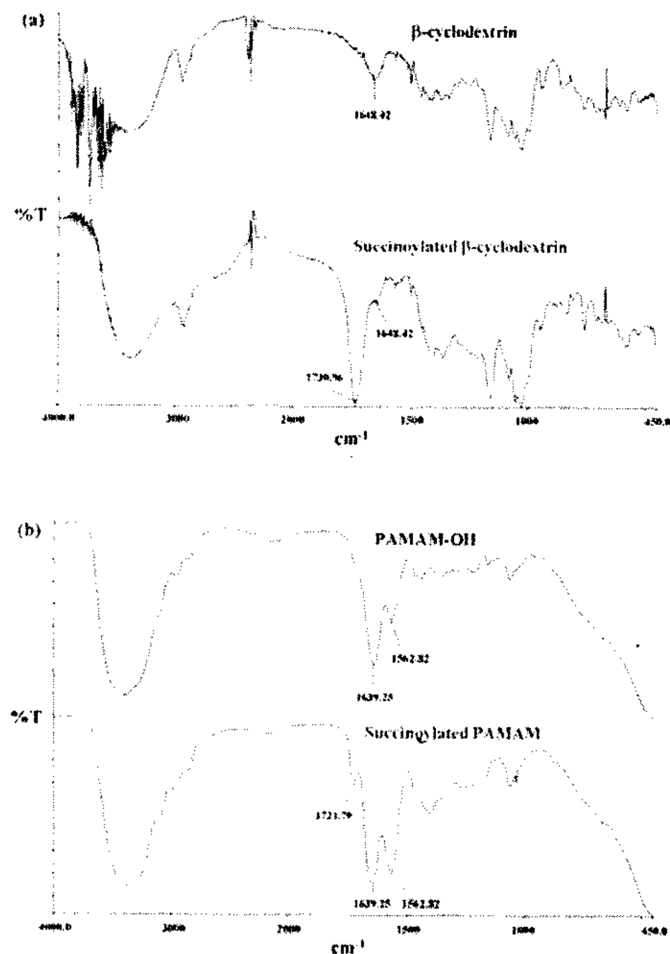

Figure 3 FT-IR characterization of (a) succinoylated β-cyclodextrin and (b) succinoylated PAMAM compare to their parent polymers.

B. *Synthesis and characterization of the polymer-trypsin conjugates*

β-cyclodextrin- and PAMAM-trypsin conjugates were prepared and purified by FPLC to separate the conjugate from free trypsin (Figure 5a). The purity was confirmed by SDS-PAGE that no free trypsin was detected (Figure 5b). The molecular weight of conjugate was predicted from FPLC standard curve as shown in Table I. The β-cyclodextrin- and PAMAM-trypsin conjugates contained 72 and 50 wt% total protein and the ratio of polymer: trypsin were 6.7:1 and 2.6:1, respectively (Table I).

TABLE I
CHARACTERISTIC OF POLYMER AND POLYMER-TRYPSIN CONJUGATES

	Protein content (wt%)	Mw (g/mol)	Polymer: trypsin ratio
Trypsin[a]	-	21,400[b]	-
β-Cyclodextrin	-	1,135	-
β-Cyclodextrin-trypsin	72	29,000[b]	6.7:1
PAMAM dendrimer	-	6,941	
PAMAM-trypsin	50	39,300[b]	2.6:1

[a]Trypsin from bovine pancreas (Mw 23,800; 223 amino acids) (http://www.sigmaaldrich.com)
[b]Estimated using protein FPLC standards (bovine pancreas ribonuclease A, Mw 13,700; chymotrypsinogen A, Mw 25,000; bovine pancreas ovalbumin, Mw 43,000 and hen egg albumin, Mw 67,000)

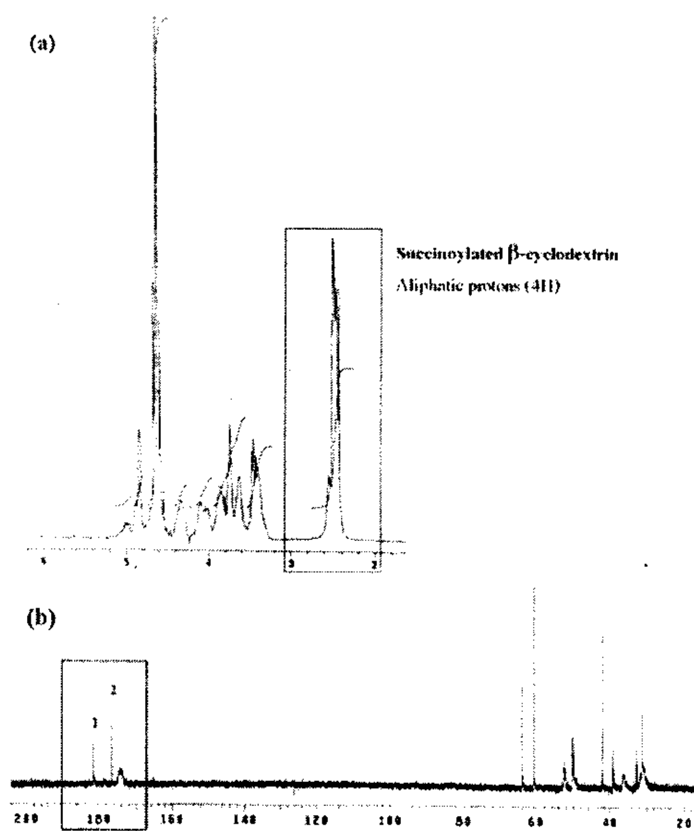

Figure 4 NMR spectra of succinoylated polymers. Panel (a) ^{1}H-NMR of succinoylated β-cyclodextrin and Panel (b) ^{13}C-NMR of succinoylated PAMAM; peak (1) showed carbonyl of carboxylic at 181.4 ppm and peak (2) showed carbonyl of ester at 176.3 ppm.

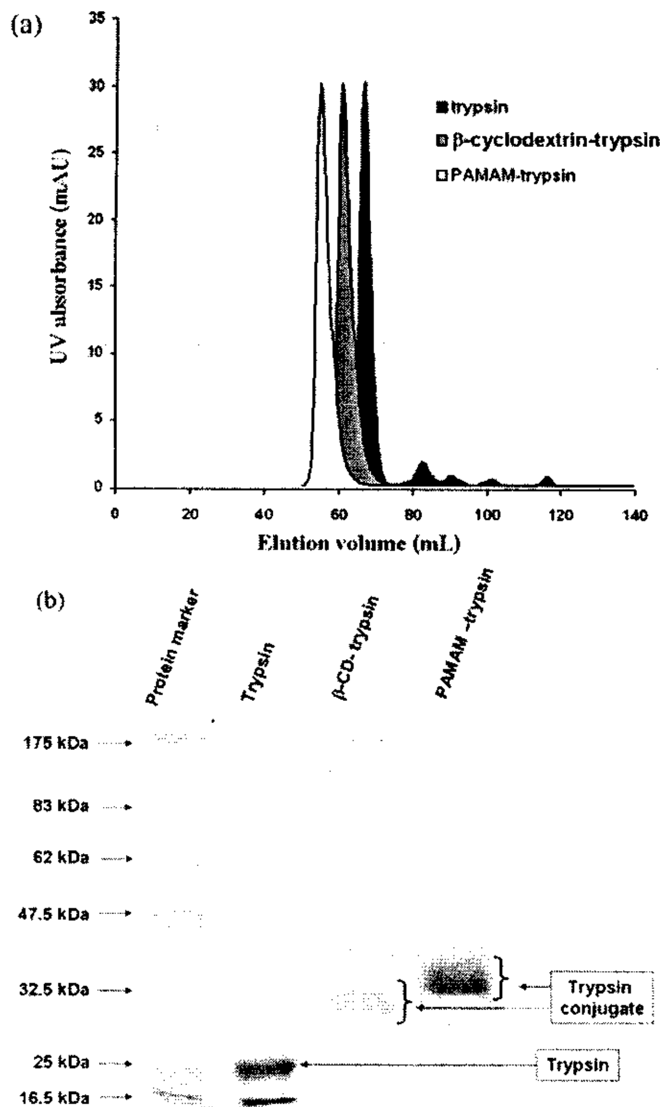

Figure 5 Characterization of β-cyclodextrin- and PAMAM-trypsin conjugate. Panel (a) FPLC and Panel (b) SDS-PAGE

C. Enzymatic activity of trypsin and polymer-trypsin conjugates

The polymer-trypsin conjugates showed lower K_M, V_{max} and K_{cat}. The activity of β-cyclodextrin and PAMAM-trypsin reduced to 13.9 and 29.2 %, respectively, compared to the free trypsin value (Table II). This result is similar to the previous studies which the reduction of trypsin activity was found after polymer conjugation [3, 23-26]. The enzymatic activity of trypsin also decreased after conjugation. It may be due to the denaturation of the protein structure during chemical modification, the modification of essential functional groups, refolding of the protein chain upon attachment of polymer, altering the active site making it less effective, or by steric hindrance preventing access of substrates to the active sites [27].

TABLE II
ENZYME ACTIVITY OF NATIVE AND POLYMER-TRYPSIN CONJUGATES

	Trypsin	β-Cyclodextrin-Trypsin conjugate[a]	PAMAM-trypsin conjugate[a]
Activity (%)	100	13.9 ± 0.7	29.2 ± 1.8
K_M(mM)	0.29 ± 0.05	0.15 ± 0.04	0.26 ± 0.02
V_{max} (μM/min)	1.5 ± 0.19	0.37 ± 0.02	0.44 ± 0.03
K_{cat}(s^{-1})	0.30 ± 0.04	0.04 ± 0.01	0.09 ± 0.01

[a]Data shown relate to the trypsin control; mean ± SD (n=3)

In this study, enzyme kinetic analysis using the Hanes-Woolfe plot indicated that Michaelis constant value (K_M) in Table II decreased after β-cyclodextrin attachment compare to native trypsin. However, K_M value was similar to native trypsin upon attachment to PAMAM, suggesting that apparent affinity is not altered by polymer conjugation. It is likely to be due to steric hindrance restricting access of the substrate to the active site. Moreover, the conjugates displayed V_{max} values that were ~3-4 fold lower, and the substrate turnover rate K_{cat} was decreased ~3-7 fold lower (Table II).

D. Thermal stability and autolysis of trypsin and polymer-trypsin conjugates

The progressive decline in enzyme activity was detected for both polymer-trypsin conjugates and trypsin with incubation time at all temperatures (30-70°C) (Figure 6a, 6b and 6c).

The β-cyclodextrin-trypsin conjugate was more stable than native trypsin at all temperatures (Figure 7a). After incubation at different temperature (30-70°C) for 10 min, it has been found that the PAMAM-trypsin conjugate was inactivated at 50°C while β-cyclodextrin-trypsin and native trypsin were inactivated at 70°C. However, thermal resistance were observed for trypsin, β-cyclodextrin-trypsin and PAMAM-trypsin conjugates, expressed in the values of T_{50} of 47°C, 55.5°C and 44°C, respectively.

Murphy and O'Fagain (1996) described the covalent modification of trypsin with acetic acid *N*-hydroxysuccinimide ethyl ester, and only an increase of 5°C was obtained for T_{50} [28]. Similarly, stabilization was also reported for trypsin modified with carboxymethylcellulose, obtaining showed increasing of 7°C for T_{50} under similar experimental conditions [25]. Several monosubstituted amino derivatives of β-cyclodextrin were also used for modifying with carboxylic group of trypsin. Fernandez, et al. (2003) were performed the similar experiment, the results showed an increase of 12.5-14.5°C for T_{50} of β-cyclodextrin-trypsin conjugates. Their results were similar to our studies which used the succinoylated β-cyclodextrin containing carboxylic end group to coupling with amino group of trypsin. The spacer arm of polymer has influenced in chemical change in the protein structure and resulted in stability effect or efficacy of polymer-protein conjugates [5].

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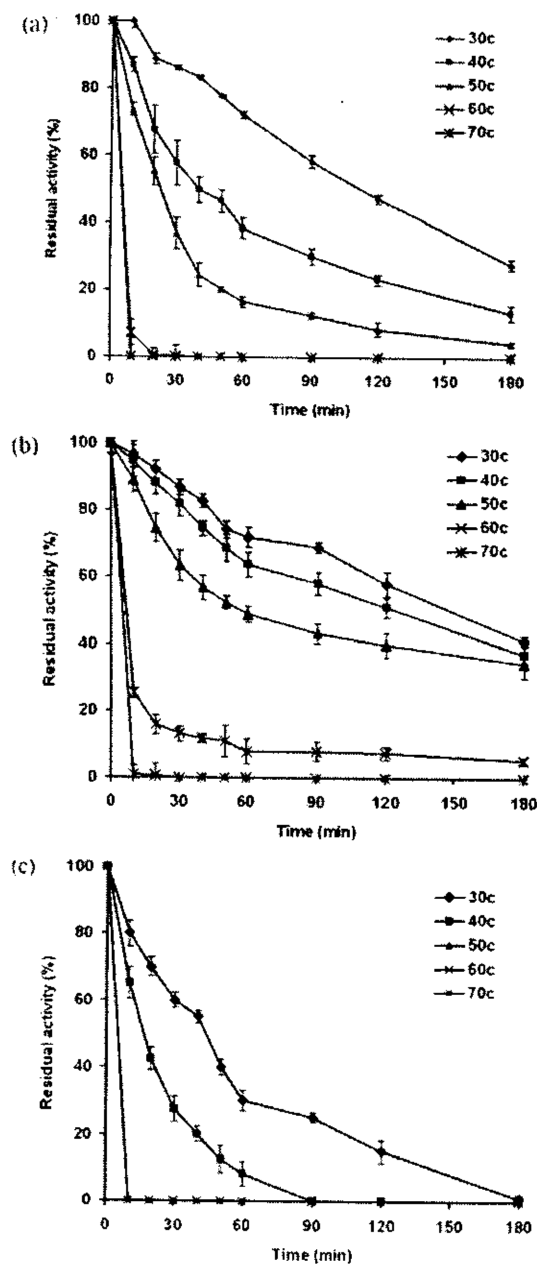

Figure 6 Effect of temperature (30-70°C) on the activity of (a) native bovine trypsin, (b) β-cyclodextrin-trypsin conjugates and (c) PAMAM-trypsin conjugates measured using BAPNA as a substrate after incubation for 180 min (mean; n = 3).

TABLE III
HALF-LIFE ($T_{1/2}$) OF NATIVE AND POLYMER-TRYPSIN CONJUGATES

Compound	Half-life ($t_{1/2}$)[a]
Trypsin	40 ± 1
β-Cyclodextrin-trypsin	125 ± 3
PAMAM-trypsin	17 ± 1
PEG-trypsin[b]	102 ± 1

[a]Experiments were conducted at 40°C and the hydrolysis of BAPNA was used to measure residual trypsin activity. Data represent t1/2 min (mean ± SD) (n=3).
[b]Data taken from [3].

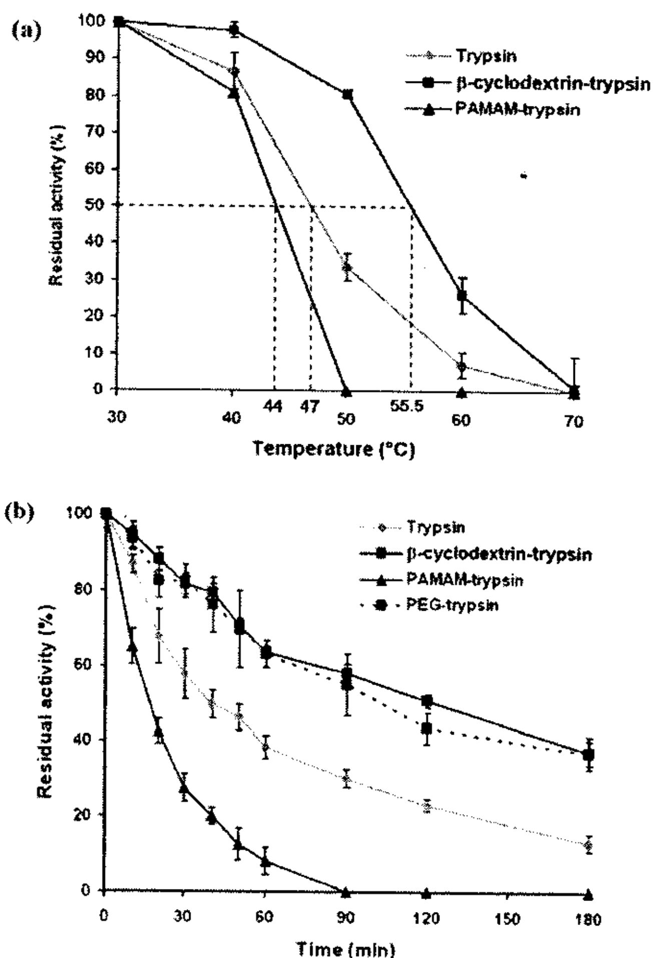

Figure 7 Autolysis (40°C) of native, β-cyclodextrin-, PAMAM- and PEG-trypsin conjugates measured using BAPNA as a substrate (mean; n = 3).

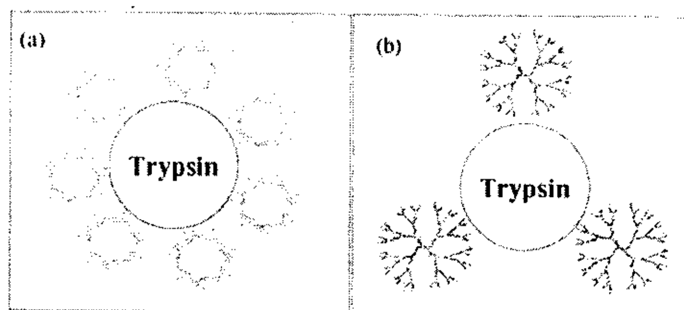

Figure 8 Possible structure of (a) β-cyclodextrin-trypsin conjugates and (b) PAMAM-trypsin conjugates

The β-cyclodextrin-trypsin was more stable while the PAMAM-trypsin conjugate was more susceptible to autolysis at 40°C than native trypsin (Figure 7b). The half-life ($t_{1/2}$) calculated from the time course for autolysis was shown in Table III. With the same linker of conjugates, the order of $t_{1/2}$ was the β-cyclodextrin-trypsin > PEG-trypsin conjugates > native bovine trypsin > PAMAM-trypsin conjugate.

The possible structure of β-cyclodextrin- and PAMAM-trypsin conjugates were shown in Figure 8. β-cyclodextrin might be expected to enhance protection from autolytic attack due to steric hindrance by wrapping around protein [3, 27]. It could be due to the glycosidic –OH groups in poly(glucose) polymer can create hydrogen-bonding to protect and stabilize proteins [28].

Interestingly, the PAMAM-trypsin conjugates displayed lower thermal stability and had a lower $t_{1/2}$ for autolysis than other conjugates and native trypsin. Trypsin was less effective after conjugated with PAMAM dendrimer. It might be because of (i) a less steric hindrance of PAMAM structure due to a less number of PAMAM molecules in the conjugate, (ii) modifying at essential functional groups that maybe interfere the active site of trypsin [27], or (iii) the chemical modification of the trypsin with PAMAM dendrimer may cause some degree of aggregation [30].

V. Conclusion

The use of β-cyclodextrin and PAMAM dendrimer as modifying agents for trypsin were described. The type and shape of polymer play important roles on kinetic parameters and enzyme activity. β-cyclodextrin had a potential to use for protein conjugation. It can stabilize and protect protein from degradation better than PAMAM dendrimer. Modifying the protein drugs with this polymer is interesting area for further investigation.

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