Journal of Applied Polymer Science

PEGylation of Deuterohaemin-Alanine-Histidine-Threonine-Valine-Glutamic Acid-Lysine and Its Influence on Activity, Stability, and Aggregation

Hang Lin,[1] Yapeng Li,[1] Hang Zhou,[1] Liping Wang,[2] Hong Cao,[3] Jun Tang,[1] Wei Li[2]

[1]College of Chemistry, Jilin University, Changchun 130012, People's Republic of China

[2]College of Life Science, Jilin University Changchun 130012, People's Republic of China

[3]Department of General Surgery, Second Hospital of Jilin University, Changchun 130041, People's Republic of China

Correspondence to: J. Tang (E-mail: chemjtang@jlu.edu.cn) or H. Cao (E-mail: caohong1967@163.com)

ABSTRACT: Deuterohaemin–alanine–histidine–threonine–valine–glutamic acid–lysine (DhHP-6) is a synthetic heme-containing peroxidase mimic that exhibits a high peroxidase enzyme activity. Compared to other microperoxidases, DhHP-6 has a poor stability and tends to aggregate in aqueous solutions. In this study, poly(ethylene glycol) (PEG) was used to improve the properties of DhHP-6. Factors that affected the PEGylation product yield were investigated. PEGylated DhHP-6 (mPEG–DhHP-6) was characterized by reversed-phase high-pressure liquid chromatography (RP-HPLC), matrix-assisted laser desorption/ionization time of flight mass spectra (MALDI-TOF-MS), and ultraviolet–visible (UV–vis) spectroscopy. The results show that the optimal PEGylation reaction conditions were achieved when the PEGylation was conducted in a borate buffer solution at pH 8.0 and 25°C for 4 h with a feeding ratio of 2 equiv of active PEG. After PEGylation, mPEG–DhHP-6 showed a great improvement in its stability with little activity loss. The UV–vis spectra of DhHP-6 and mPEG–DhHP-6 in different pH solutions showed that the aggregation of DhHP-6 was partly suppressed after PEGylation. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: biomaterials; peptides; UV–vis spectroscopy

Received 2 September 2011; accepted 16 June 2012; published online
DOI: 10.1002/app.38220

INTRODUCTION

Heme-containing peroxidases are important enzymes and have been widely studied because they can catalyze the oxidation of a variety of compounds with hydrogen peroxide or hydrogen peroxide related compounds as oxidants.[1–3] Microperoxidases are heme peptides obtained by the proteolytic digestion of cytochrome c and display heme-containing peroxidase activity.[4] However, most heme-containing peroxidases and microperoxidases are expensive; this limits their wide application. Recently, a heme-containing microperoxidase mimic, deuterohaemin–alanine–histidine–threonine–valine–glutamic acid–lysine (DhHP-6, shown in Figure 1), was synthesized in our laboratory.[5] In this microperoxidase mimic, two vinyl substituents were removed from heme to improve its stability against its peroxide substrates. The six-amino-acid peptide derived from microperoxidase 9 provides the enzyme activity of DhHP-6. DhHP-6 exhibits high peroxidase activity, which can protect cultured rat lens crystallina from cataracts induced by galactose, enhance the antioxidant enzyme activity in *Caenorhabditis elegans*, and prevent hydrogen peroxide induced cell damage in H9c2 cells.[6–8] DhHP-6 can also be used for the removal of phenol or catalyzing polymerization (unpublished data). In addition, DhHP-6 is much cheaper than classical heme-containing peroxidases and their microperoxidases.[6,9]

PEGylation is defined as the chemical modification of proteins and peptides with poly(ethylene glycol) (PEG). It has been used as a feasible approach for improving the properties, such as stability, solubility, and aggregation behavior, of proteins and peptides.[10–14] Al-Azzam et al.[15] reported decreased aggregation and improved stability in horseradish peroxidase (HRP) after PEGylation. Like other microperoxidases, the hydrophobic porphyrin ring of DhHP-6 tends to form aggregates in aqueous solutions.[16] A PEGylation strategy has been adopted to reduce the aggregation of DhHP-6 and improve its stability. The most common route for the PEG conjugation of peptides is to use activated PEG with functional groups suitable to react with lysine residues and *N*-terminal amino acid groups in various water-based buffer solutions.[17,18] Although a general

Additional Supporting Information may be found in the online version of this article.

Applied Polymer SCIENCE

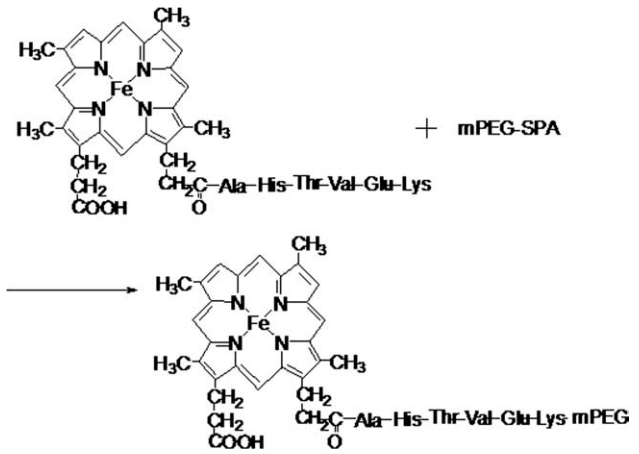

Figure 1. Structure of DhHP-6 and synthetic route of the mPEG–DhHP-6 synthesis.

modification procedure can adopted for PEGylation, because of the presence of different types of proteins and peptides, the conjugation conditions for each protein and peptide will be varied; thus, a significant effort would be required to determine the optimal coupling conditions. Both carboxyl and amino groups in DhHP-6 can be used for PEGylation. In this study, the ϵ-NH_2 group in lysine residue was chosen as the coupling site because the amino group is more reactive than the carboxyl group. Furthermore, because of the presence of two carboxyl groups in DhHP-6, the use of the carboxyl as the coupling site would make the characterization and purification of the PEGylation reaction more difficult. α-Methoxy-ω-succinimidyl propionate–poly(ethylene glycol) (mPEG–SPA) was chosen as the coupling agent. The PEGylation reaction was conducted at different pH values, temperatures, times, feeding ratios, and so on. The optimal reaction conditions were determined with product yield as the selection criteria. The stability and enzyme activity of DhHP-6 and PEGylated DhHP-6 (mPEG–DhHP-6) were studied. Finally, the ultraviolet–visible (UV–vis) spectra of DhHP-6 and mPEG–DhHP-6 were compared.

EXPERIMENTAL

Materials

DhHP-6 was synthesized in our laboratory as previously described.[9] mPEG–SPAs, with number-average molecular weights of 5 and 20 kDa, were purchased from Beijing JenKem Technology Co., Ltd. All other chemicals and reagents were analytical grade and were purchased from Sinopharm Chemical Reagent Beijing Co., Ltd.

Preparation and Characterization of the mPEG–DhHP-6

DhHP-6 (0.1 mg) was dissolved in 1 mL of phosphate-buffered saline (PBS; 0.05*M*, pH = 7.0, 7.5, and 8.0) or borate solution (0.05*M*, pH = 7.5, 8.0, and 9.0). Activated PEG powder (1.2, 2, and 5 equiv to DhHP-6) was then added to the peptide solution. PEGylation was allowed to proceed at different temperatures for 4–24 h. The reaction solution was purified by dialysis or reversed-phase high-pressure liquid chromatography (RP-HPLC), and the solvent was removed by lyophilization. The PEGylation reaction solution was characterized by RP-HPLC (Agilent 1100 series instrument, Palo Alto, CA) on Edipse XDB-C_{18} (4.6 × 250 mm^2, 5 μm) at room temperature. The mobile phase consisted of 0.1% trifluoroacetic acid (TFA) in distilled water (eluent A) and acetonitrile (ACN) containing 0.1% TFA (eluent B). The mobile phase was run with a linear gradient from 30 to 60% of eluent B for 15 min at a flow rate of 1 mL/min for analysis. The UV absorbance of the eluent was monitored at 386 nm. The RP-HPLC fractions corresponding to the respective peaks were collected separately, purged with nitrogen, and then lyophilized. The molecular mass of different elution peaks was obtained from matrix-assisted laser desorption/ionization time of flight (MALDI–TOF) mass spectrometry (MS) with Kratos AX1MA-CFR MALDI–TOF MS (Shimadz Co., Kyoto, Japan). Samples were prepared by the mixture of 1 equiv of the aliquot with 2 equiv of the matrix solution, a saturated solution of α-cyanohydroxycinnamic acid in 50% water/acetonitrile (ACN) with 0.3% TFA. The data obtained from 2-ns pulses of a 337-nm nitrogen laser were averaged for each spectrum in the reflection mode, and positive-ion TOF detection was performed with an accelerating voltage of 25 kV. NMR spectra were obtained with a Bruker AVANCE500 (Bruker, Rheinstetten, Germany) spectrometer. All samples were dissolved in D_2O. The KCN and peptide complexes were prepared by the addition of concentrated solutions of ligands to the NMR tubes. All experiments were conducted at 300 K.

Enzyme Activity

DhHP-6 (100 μL, 0.1 mg/mL in buffer solution), vitamin C (100 μL, 5 μmol/mL in buffer solution), and buffer solution (700 μL) were mixed and incubated in a water bath at 37°C for 5 min. Then, 100 μL of H_2O_2 (1 mmol/mL in buffer solution) was added to this mixture. The absorbance changes over time were monitored by UV–vis spectroscopy at 290 nm and 25°C for 5 min. The enzyme activities in the pH range 5.0–9.0 were determined with buffer solutions with different pH values. mPEG–DhHP-6 was treated under the same conditions. The activity was proportional to the rate of consumption of the vitamin C, which absorbed light at 290 nm, with an extinction coefficient of 400 mL g^{-1} cm^{-1}. One unit of activity (U) was defined as the amount of enzyme required to oxidize 1 μg of vitamin C per minute at 25°C and pH 7.0.

Papain Digestion

Papain (50 μL, 0.1 U/mL in PBS, 7.4, 0.05*M*) was added to mPEG–DhHP-6 at a concentration of approximately 2 mg/mL in 450 μL of PBS. Proteolytic digestion was carried out at 40°C for 0.5 h. Unmodified DhHP-6 was treated under the same conditions as a control. The papain digestion was monitored with RP-HPLC with the previously described parameters. The RP-HPLC fractions corresponding to the respective peaks were collected separately, purged with nitrogen, and then characterized by MALDI–TOF MS.

UV–Vis Spectroscopy

The UV–vis absorption spectra were obtained with a Shimadzu UV-2501 spectrophotometer. DhHP-6 or mPEG–DhHP-6 was dissolved in 0.01*M* HCl, NaOH, or 0.05*M* buffer solutions (acetate, phosphate, or borate) with various pH values. The ionic strength was adjusted with 0.10*M* KCl. The concentrations of all

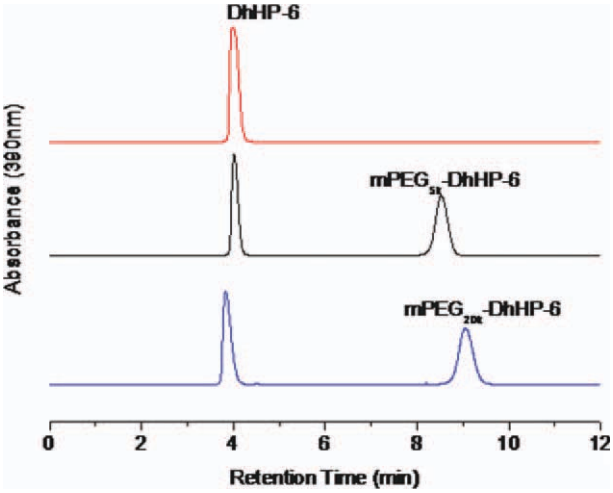

Figure 2. RP-HPLC spectra of the aqueous solution of DhHP-6, the reaction solution of $mPEG_{5k}$–DhHP-6, and the reaction solution of $mPEG_{20k}$–DhHP-6. Both reactions were conducted in borate buffer (8.0, 0.05*M*) at 25°C with a feeding ratio of 1 : 1. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

of the solutions were maintained at 0.1 μmol/mL. Statistical analysis was carried out with SPSS software (version 17, Chicago, IL).

RESULTS AND DISCUSSION

Characterization of mPEG–SPA with DhHP-6

PEGylation is a feasible way to improve the properties of proteins and peptides but still maintain their activities. RP-HPLC and MALDI–TOF MS are commonly used to characterize the PEGylation reaction.[19,20] In our study, RP-HPLC was used to monitor the extent of PEGylation (Figure 2). The retention time of DhHP-6 was 4.0 min. A new elution peak with a retention time of 8.0 min appeared in the reaction solution of DhHP-6 and mPEGSPAs, with number-average molecular weights of 5000 Da ($mPEG–SPA_{5k}$). A portion of the reaction solution of this new peak was collected and characterized by MALDI–TOF MS. $mPEG–SPA_{5k}$ was also characterized for comparison. As shown in Figure 3(a), the molecular masses were 4774 Da for $mPEG–SPA_{5k}$ and 5887 Da for the new elution peak. Even though only the midpoint values of the molecular mass range were determined by MALDI–TOF MS because of the polydispersity of PEG, with the molecular mass of DhHP-6 being 1229 Da, the obtained mass of the conjugates was close to the sum of the masses of the individual components of PEG-modified DhHP-6, and the small deviation was within the range of error. These results indicate that the new peak was from mono-mPEG–DhHP-6. It should be noted that the broad MALDI–TOF MS peak appearing after PEGylation was probably due to the presence of unreacted PEG. The ^{1}H-NMR spectra of DhHP-6, $mPEG_{5k}$–SPA, and $mPEG_{5k}$–DhHP-6 are shown in the Supporting Information (Figure S1). After PEGylation, a few broad peaks appeared between 10 and 22 ppm in the ^{1}H-NMR spectrum of $mPEG_{5k}$–DhHP-6. These new peaks were attributed to interactions between the hydrogen atoms of PEG and the porphyrin core. Further confirmation of these peaks and the proton assignments of DhHP-6 and mPEG–DhHP-6 is still being attained in our laboratory. In the case of mPEG-DhHP-6 with a PEG of number-average molecular weights of 20000 Da ($mPEG_{20k}$–DhHP-6), a new elution peak appeared at 9.0 min [Figure 3(b)]. The molecular masses obtained from MALDI–TOF MS were 19,058 Da for $mPEG_{20k}$ and 20,228 Da for $mPEG_{20k}$–DhHP-6.

Many factors can affect the product yield of the PEGylation reaction. Here, the effects of different pHs on the production yield of the PEGylation of DhHP-6 were investigated, first in aqueous buffer solutions with pHs ranging from 7.0 to 9.0 with an interval of 0.5. The results are shown in Figure 4. At pH 7.0, the yield of $mPEG_{5k}$–DhHP-6 was around 37%. The yields of the PEGylation reactions increased as the pH of the aqueous buffer solution was increased. When the pH reached 8.0, a plateau appeared, and the product yield was maintained at around 50% throughout the pH range 8.0–9.0. Actually, the product yield decreased slightly from about 53% at pH 8.0 to 51% at pH 9.0. It should be noted that two types of buffer solutions were used in this study. The first type was a borate buffer solution having a pH above 8.0, whereas the second type was a PBS

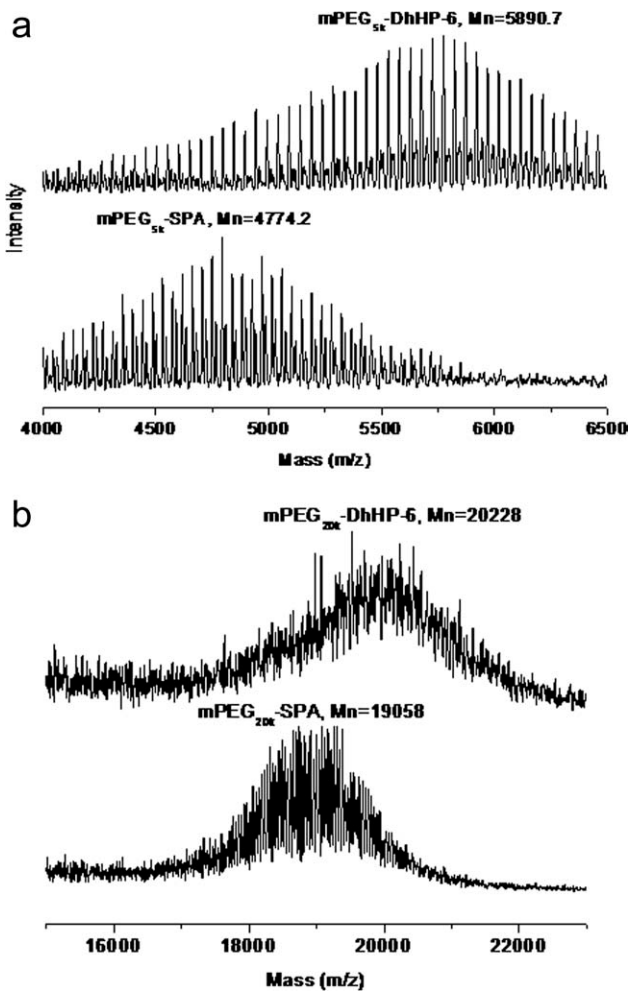

Figure 3. MALDI–TOF MS spectra of (a) $mPEG_{5k}$–DhHP-6 and $mPEG_{5k}$–SPA and (b) $mPEG_{20k}$–DhHP-6 and $mPEG_{20k}$–SPA.

Applied Polymer SCIENCE

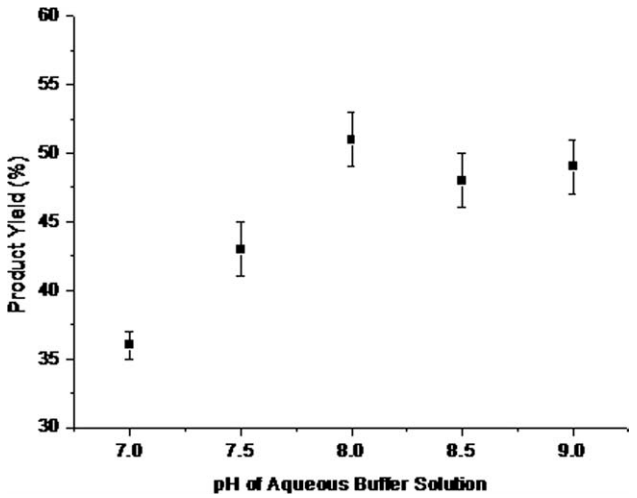

Figure 4. Product yield of mPEG$_{5k}$–DhHP-6 in aqueous buffer solutions with different pH values.

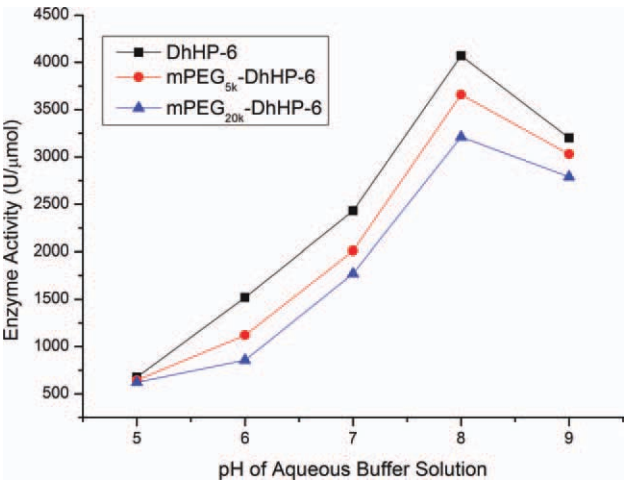

Figure 6. Enzyme activities of DhHP-6 and mPEG–DhHP-6 in aqueous buffer solutions with different pH values. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

buffer solution having a pH below 8.0. To investigate whether the increase in the product yield at higher pH was due to the different types of buffer solution, PEGylation was also conducted in borate buffer solutions having pH values of 7.5 and 8.0. Product yields of mPEG$_{5k}$–DhHP-6 in different buffer solutions did not show a significant difference. These results suggested that the types of aqueous buffer solutions did not affect the product yield.

The temperature is an important factor in chemical reactions and affects the rate of reaction. Therefore, PEGylations were conducted at different temperatures (4, 25, and 30°C) in borate buffer at pH 8.0. The product yield was only 18% at 4°C, even after 24 h of reaction. This was because the reaction rates were significantly slower at low temperatures, as explained in Morpurgo and Veronese's report.[18] The yield of mPEG$_{5k}$–DhHP-6 increased with increasing reaction temperature. The yields increased to 53% at 25°C and 57% at 30°C after 4 h of reaction. Because the enzyme stability tended to be reduced at elevated temperatures, we did not test the product yield at temperatures above 30°C, because a significant improvement in the product yield was observed in PEGylation when the temperature was increased from 25 to 30°C.

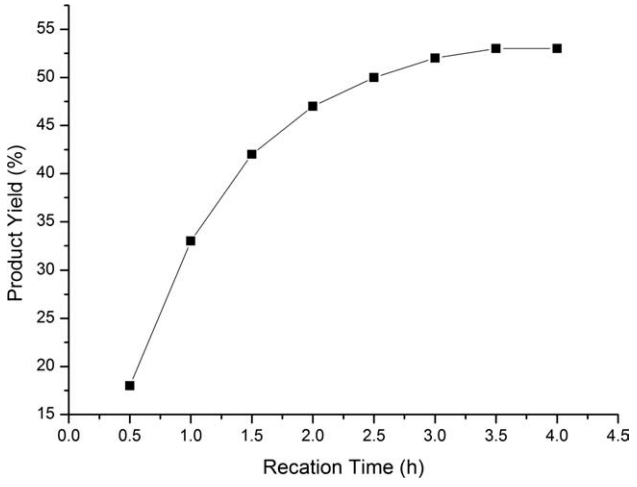

Figure 5. Relationship between the time and product yield of mPEG$_{5k}$–DhHP-6.

The effect of the reaction time on the PEGylation product yield was studied (Figure 5). PEGylation was conducted in a borate buffer solution at pH 8.0 and 25°C. The reaction solution was examined by RP-HPLC every 0.5 h during the 4-h reaction time. About 47% of mPEG$_{5k}$–DhHP-6 was obtained after 2 h of reaction. The maximum yield was reached after 3.5 h. Afterward, the solution was reacted for another 0.5 h to ensure the completion of the reaction.

On the basis of the previous results, PEGylation was conducted under the optimal conditions, that is, in a borate buffer solution at pH 8.0 and 25°C for 4 h. With a feeding ratio of 1 : 1.2, the yield of mPEG–DhHP-6 was 53% for mPEG–SPA$_{5k}$ and 49% for mPEG–SPA$_{20k}$. Although the product yield decreased with increasing molecular mass of PEG, the decrease was much smaller than expected. This might have been due to the easy accessibility of the coupling site in DhHP-6. In the PEGylation of DhHP-6, because the reaction site was ϵ-NH$_2$ of Lys, which is located in the distal end of the enzyme mimic, the steric hindrance in DhHP-6 was not severe enough to prevent the larger size PEG from approaching the ϵ-NH$_2$ coupling site.

A higher product yield could be achieved through an increase in the feeding ratio of activated PEG. For mPEG$_{5k}$–SPA, the yields were 75% for a feeding ratio of 1 : 2 and 95% for a feeding ratio of 1 : 5. Although a higher feeding ratio gave a higher product yield, it was difficult to separate mPEG–DhHP-6 and the excess amount of unreacted mPEG, especially when the PEG molecular mass was higher than 20 kDa. Therefore, the feeding ratio of 1 equiv of DhHP-6 versus 2 equiv of activated PEG should have been the optimal feeding ratio in our case.

Enzyme Activities of DhHP-6 and mPEG–DhHP-6

Activity is important for any enzyme. However, PEGylation always results in an enzyme activity loss. Here, the enzyme

activities of DhHP-6 and mPEG–DhHP-6 with different molecular weights were detected (Figure 6). All samples showed almost the same low activities around 600 U/μmol when the pH value was 5.0. The enzyme activities of DhHP-6 and mPEG–DhHP-6 increased with increasing pH until the pH value reached 8.0. This indicated that the maximum enzyme activity of DhHP-6 occurred at pH 8.0. The maximum enzyme activity of DhHP-6 was 4070 U/μmol; this was in accordance with previously reported results.[6] The maximum enzyme activity of mPEG–DhHP-6 also occurred at pH 8.0. The activity decreased increasing molecular weight of PEG, from 3660 U/μmol for $mPEG_{5k}$–DhHP-6 to 3210 U/μmol for $mPEG_{20k}$–DhHP-6. Further increases in the pH of the solutions resulted in a decrease in the activities for all samples. It should be noted that the enzyme activities decreased with increasing molecular weight of conjugated mPEG at all pH values. The decrease in the enzyme activity was probably due to the steric hindrance of PEG. Although PEGylation indeed lowered the DhHP-6 activity, more than 80% of enzyme activity of DhHP-6 was still maintained for mPEG–DhHP-6's with different molecular weights. Therefore, the study of the enzyme activity of DhHP-6 indicated that the PEGylation of DhHP-6 had little effect on the enzyme activity of DhHP-6.

Stabilities of DhHP-6 and mPEG–DhHP-6 under Papain Digestion

PEGylation can improve the solubility and stability of peptides and proteins. The solubility difference between DhHP-6 and mPEG–DhHP-6 is described in another section. Papain digestion was used to investigate the improvement of the stability of mPEG–DhHP-6, and the results are presented here. The RP-HPLC spectra of the papain digestion of DhHP-6, $mPEG_{5k}$–DhHP-6, and $mPEG_{20k}$–DhHP-6 for 0.5 h are shown in Figure 7(a). A new elution peak at 6.6 min appeared for every tested sample. The molecular mass of this peak obtained from MALDI–TOF MS analysis was 1101. This value corresponded to the molecular mass of DhHP-5 obtained after lysine was hydrolyzed from DhHP-6. After digestion for 0.5 h, 70% of DhHP-6 was hydrolyzed, whereas 95.4% of $mPEG_{5k}$–DhHP-6 and 96.9% of $mPEG_{20k}$–DhHP-6 still remained. This indicated that the PEGylation of DhHP-6 significantly increased the stability of DhHP-6 against papain.

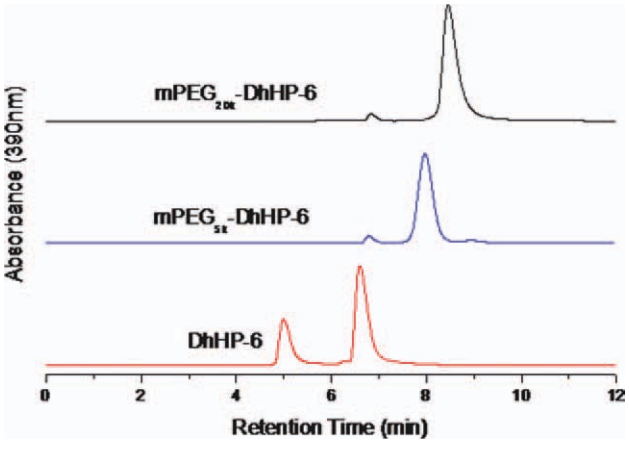

Figure 7. RP-HPLC spectra of the papain digestion solution. Elution peaks with retention times of 4.0, 6.6, 8.0, and 9.0 min were identified as DhHP-6, DhHP-5, $mPEG_{5k}$–DhHP-6, and $mPEG_{20k}$–DhHP-6, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

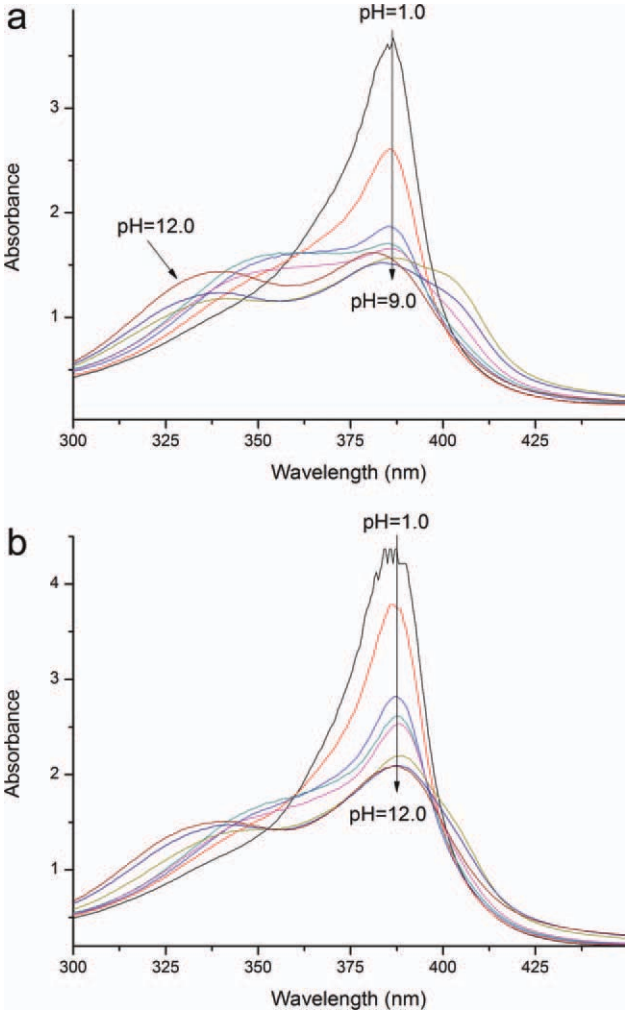

Figure 8. UV–vis spectra of (a) DhHP-6 and (b) $mPEG_{20k}$–DhHP-6 in aqueous buffer solutions with pH values ranging from 1.0 to 12.0. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Influence of PEGylation on the UV–Vis Spectra

Because the UV–vis spectra of the water-soluble hemins disclose important information about the aggregation and coordination states,[21–23] the UV–vis spectra of DhHP-6 and mPEG–DhHP-6 were investigated. The absorption spectra of water-soluble hemins are affected by many factors, including pH, concentration, and ionic strength. Among these factors, pH is one of the most sensitive factors that affect heme aggregation. Therefore, the effect of PEGylation at different pH values on the aggregation of PEGlated–DhHP6 was studied intensively by UV–vis spectra (Figure 8). The effects of the pH values on the UV–vis spectra of mPEG–DhHP-6 with different molecular masses were similar; thus, only the UV–vis spectra of $mPEG_{20k}$–DhHP-6 are described here, as an example. To investigate the influence of residue-free PEG on the UV–vis spectra of DhHP-6, excesses of different molecular weights of free PEG of up to 10 times were

Table I. N/S Ratios of DhHP-6 and $mPEG_{20k}$–DhHP-6 at Different pH Values

Solution pH	5.0	6.0	7.0	8.0	9.0	12.0
DhHP-6	0.862	0.947	0.880	0.752	0.815	0.889
$mPEG_{20k}$–DhHP-6	0.596	0.659	0.641	0.665	0.704	0.723

added to the DhHP-6 solution. T No obvious spectra changes were observed in UV–vis spectra.

As shown in Figure 8(a), DhHP-6 exhibited a Soret band at 385 ± 1 nm at pHs ranging from 1.0 to 8.0. The intensity of the Soret-band absorption decreased with increasing pH. The Soret band was blueshifted when the pH increased. However, the maximum absorbance wavelength of the Soret band of $mPEG_{20k}$–DhHP-6 was not affected by the pH values and was 388 ± 0.5 nm. In contrast, the Soret band was redshifted for $mPEG_{20k}$–DhHP-6 at all pH values. This indicated that the extent of nonplanar distortion of the heme porphyrin ring increased after PEGylation.[24] An obvious shoulder peak appeared at 403 nm in the UV–vis spectrum of DhHP-6 when the pHs of the solution were 8.0 and 9.0. This shoulder peak was attributed to a high activity state of DhHP-6. After PEGylation, intensity of this shoulder peak was weak, and this resulted in enzyme activity loss. When the pH value was higher than 5.0, an N band around 350 nm appeared in the UV–vis spectra of both DhHP-6 and $mPEG_{20k}$–DhHP-6. As the pH of the solution increased, the absorbance of the N band also increased; however, the absorbance of the Soret band decreased. Here, the values of the ratio between the absorbance of the N band and that of the Soret band (N/S) for DhHP-6 and $mPEG_{20k}$–DhHP-6 at different pH values were used to denote the relative degree of aggregation (Table I). Because Munro and Marques[25] concluded that UV–vis spectral changes below pH 4.0 did not reflect the aggregation of the chromophore, only the spectral changes above 4.0 are considered here. When the pH value was 5.0 or higher, N/S of mPEG–DhHP-6 was obviously lower than that of DhHP-6. An independent-sample *T* test was used for statistical analysis, and a *p* value of 0.008 was obtained. This indicated that there was a significant difference in the N/S values between DhHP-6 and $mPEG_{20k}$–DhHP-6. These results suggest that PEGylation significantly decreased the aggregation of DhHP-6. This reduced aggregation was attributed to the decreased amount of ϵ-NH_2 in the lysine residue of DhHP-6 and the steric hindrance of conjugated PEG.

CONCLUSIONS

In this article, a heme-containing microperoxidase mimic, DhHP-6, was PEGylated. The PEGylation reaction conditions were investigated to determine the optimal reaction conditions. The reaction conducted in a borate buffer solution at pH 8.0 and 25°C for 4 h with the feeding 2 equiv of active PEG gave a relatively high product yield, and these conditions were considered to be optimal. Although a higher feeding ratio resulted in a higher yield, the difficulty in removing excess PEG and the associated high cost made feeding ratios higher than 2 less favorable. Enzyme activity assays showed that the maximum enzyme activities for both DhHP-6 and mPEG–DhHP-6 occurred at pH 8.0 PEGylation also greatly improved the stability of DhHP-6 with little activity loss. The UV–vis spectra of DhHP-6 and mPEG–DhHP-6 at different pHs showed significant differences and indicated that the PEGylation of DhHP-6 greatly suppressed the aggregation of DhHP-6

ACKNOWLEDGMENTS

Financial support was provided by the Open Project of the State Key Laboratory for Supramolecular Structure and Materials (grant number SKLSSM201003) and Jilin Province Science & Technology Pillar Program (grant numbers 20080933 and 20110336).

REFERENCES

1. Kobayashi, S.; Makino, A. *Chem. Rev.* **2009,** *109*, 5288.
2. Obinger, C. *Arch. Biochem. Biophys.* **2010,** *500*, 1.
3. Dunford, H. B. Heme Peroxidases, Wiley:New York, **1999.**
4. Kadnikova, E. N.; Kostic, N. M. *J. Org. Chem.* **2003,** *68*, 2600.
5. Liu, Y.; Guo, L. L.; Roeske, W. R.; Luo, G. M.; Li, W. *Acta. Sci. Nat. Univ. Jilin* **2001,** *1*, 91 (in Chinese).
6. Wang, L. P.; Liu, Y. L.; Yang, H.; Li, W. *Chem. J. Chin. Univ.* **2004,** *25*, 2171 (in Chinese).
7. Wang, L. P.; Guan, S. W.; Li, P. F.; Luo, J.; Li, Y. Y.; Huang, L.; Wang, G. A.; Zhu, L. M.; Fan, H. K. A.; Li, W. *Free Radical Res.* **2010,** *44*, 813.
8. Guan, S. *J. Pept. Sci.* **2010,** *16*, 133.
9. Du, H. D.; Yan, Y. M.; Wang, L. P.; Li, M. *J. Pept. Sci.* **2008,** *14*, 105.
10. Veronese, F. M.; Harris, J. M. *Adv. Drug Delivery Rev.* **2008,** *60*, 1.
11. Harris, J. M.; Chess, R. B. *Nat. Rev. Drug Discovery* **2003,** *2*, 214.
12. Roberts, M. J.; Bentley, M. D.; Harris, J. M. *Adv. Drug Delivery Rev.* **2002,** *54*, 459.
13. Veronese, F. M. *Biomaterials* **2001,** *22*, 405.
14. Jevsevar, S.; Kunstelj, M.; Porekar, V. G. *Biotechnol. J.* **2010,** *5*, 113.
15. Al-Azzam, W.; Pastrana, E. A.; King, B.; Mendez, J.; Griebenow, K. *J. Pharm. Sci.* **2005,** *94*, 1808.
16. Melchionna, S.; Barteri, M.; Ciccotti, G. *J. Phys. Chem.* **1996,** *100*, 19241.
17. Fee, C. J.; Van Alstine, J. A. *Chem. Eng. Sci.* **2006,** *61*, 924.
18. Morpurgo, M.; Veronese, F. M. *Methods Mol. Biol.* **2004,** *283*, 45.
19. Wang, X.; Tian, Y.; Lin, H.; Zang, L. *J. Appl. Polym. Sci.* **2010,** *116*, 3220.
20. Zhou, K.; Zheng, X.; Xu, H. M.; Zhang, J.; Chen, Y.; Xi, T.; Feng, T. *Bioconjug. Chem.* **2009,** *20*, 932.
21. Othman, S.; Le Lirzin, A.; Desbois, A. *Biochemistry* **1993,** *32*, 9781.
22. Aron, J.; Baldwin, D. A.; Marques, H. M.; Pratt, J. M.; Adams, P. A. *J. Inorg. Biochem.* **1986,** *27*, 227.
23. Harbury, H. A.; Loach, P. A. *J. Biol. Chem.* **1960,** *235*, 3640.
24. Sparks, L. D.; Medforth, C. J.; Park, M. S.; Chamberlain, J. R.; Ondrias, M. R.; Senge, M. O.; Smith, K. M.; Shelnutt, J. A. *J. Am. Chem. Soc.* **1993,** *115*, 581.
25. Munro, O. Q.; Marques, H. M. *Inorg. Chem.* **1996,** *35*, 3752.