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Large-scale production of *N*,*N*′-diBoc-dityrosine and dityrosine by HRP-catalyzed *N*-Boc-L-tyrosine oxidation and one-step chromatographic purification

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ABSTRACT

Dityrosine (DY) can be used as a biomarker to detect oxidative protein damage and selective proteolysis. It is generally prepared by horseradish peroxidase (HRP)-catalyzed oxidation of L-tyrosine (Y) followed by multistep chromatographic separations. In this study, we present an alternative method for the preparation of DY by HRP-catalyzed synthesis of *N*,*N*′-diBoc-dityrosine (DBDY) from *N*-Boc-L-tyrosine (BY). The presence of the *tert*-butoxycarbonyl (Boc) group ensured that the fraction of further oxidized by-products (e.g., trimers and pulcherosine) was quite low. The yield of DBDY (37.5%) was comparable to that reported for DY (> 26%). DBDY could be purified by a simple one-step silica column chromatography procedure that resulted in a purity of 89.7%. DBDY is considered to be better than DY for subsequent chemical reactions (for binding to polymers, amino acids, drugs, antibodies, etc.) because such reactions can be selectively performed by using the carboxylic acid and amine groups in the following sequence: first, the carboxylic acid groups are used; next, the Boc groups are removed; and finally, the amino groups are used. To prepare DY, the Boc groups in DBDY were simply and completely removed by treatment with trifluoroacetic acid.

1. Introduction

Dityrosine (DY) is formed when proteins are exposed to reactive oxidizing agents (e.g., hydrogen peroxide, hydroxyl radical, and superoxide radical), ionizing radiation, or peroxidase, and it is associated with Alzheimer's and Parkinson's diseases [1–3], cystic fibrosis [4], and atherosclerosis [5]. Yoburn et al. [6] reported that DY formation in γ_b-crystallin, calmodulin, ribonuclease A, and bovine serum albumin resulted in protein denaturation and enhanced precipitation.

DY can be used as a biomarker to detect oxidative protein damage and selective proteolysis [7–9]. DY occurs naturally in fungal cell-wall proteins, insect eggs, and sea-urchin envelopes as well as in extensible proteins such as elastin, resilin, and calmodulin [10–14]. In these proteins, DY plays the role of a crosslinking agent and can help in increasing the mechanical strength. To adequately measure DY formation in biological samples, an efficient method is required to produce this compound in sufficient quantities [15]. There has been an attempt to produce novel polymeric materials in which DY functions not only as the crosslinking agent but also as the fluorescent core [16]. The above discussion indicates that there is a growing need for the large-scale production of DY.

Several methods have been reported for the preparative-scale synthesis of DY. Nonenzymatic preparation methods involving the use of oxidizing agents such as potassium ferricyanide, sodium persulfate, and ferrichloride have been reported. However, the yield was less than 5% in these cases [17,18], and large amounts of higher oxidation products such as trityrosine and isotrityrosine were inevitably formed [18]. Several elegant but complicated schemes have been suggested for producing DY in high yields. For example, Nishiyama et al. [19] successfully obtained a yield of 28% by protection of the amino and carboxylic groups of tyrosine, iodination at both the *ortho* positions of the phenol group, oxidative electrolysis, zinc reduction, catalytic hydrogenation, and acidic hydrolysis. Hutton and Skaff [20] succeeded in obtaining a yield of 39% by employing the following series of reactions: protection of the amino and carboxylic groups of tyrosine, iodination at the *ortho* position of a phenol group, Miyaura borylation, and Suzuki coupling. However, high-level skills and experience of organic synthesis are required to attain this level of productivity. We have earlier reported a simple one-step synthesis reaction for DY preparation using Mn(III) as the oxidizing agent [21]. However, a common feature of these chemical preparation methods described above is that several purification steps such as precipitation, filtration, and chromatographic separation are necessary because various impurities are present in addition to DY.

Enzymatic preparation of DY generally employs a peroxidase to catalyze the oxidative coupling of Y, and a yield of more than

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26% has been achieved with this method [22]. However, due to the zwitterionic property of DY and the presence of by-products such as isodityrosine, trityrosine, and pulcherosine, at least two chromatographic separation steps (e.g., gel filtration chromatography, ion exchange chromatography, and thin-layer chromatography) are required in this process [18,22].

The purpose of this study was to develop an alternative method for the enzymatic preparation of DY. Similar to other enzymatic methods, horseradish peroxidase (HRP) was chosen as the model peroxidase. *N*-Boc-L-tyrosine (BY) was used as the substrate, and *N*,*N*′-diBoc-dityrosine (DBDY) was the expected product. As DBDY does not have a free amino group, it is not a zwitterion.

Similar to our study, Amado et al. [18] used *N*-acetyl-L-tyrosine (AY) as the substrate of HRP and synthesized *N*,*N*′-diacetyl-dityrosine (DADY). Deblocking (i.e., removal of acetyl groups) was performed by hydrolysis in 6 M HCl solution under N_2 reflux for 20 h. Hydrolysis under such harsh conditions resulted in several impurities and affected the subsequent reactions of DY (e.g., amide bond formation between DY and other materials) [23]. Consequently, three chromatographic separation steps had to be employed in this process, which was similar to the process in which Y was used as the HRP substrate [18]. The Boc group used to protect the amino group is generally removed by hydrolysis with trifluoroacetic acid (TFA) in methylene chloride for 30 min at room temperature and 1 atm [24–27]. The resulting Boctrifluoroacetate and residual TFA are easily removed without additional chromatography steps.

2. Materials and methods

2.1. Chemicals

N-Boc-L-tyrosine (98%), L-tyrosine (99%), HRP, boric acid (99%), sodium tetraborate decahydrate (99.5%), TFA (99%), *N*,*N*-dimethylformamide (DMF) (99.8%), dimethyl sulfoxide-d_6 (DMSO-d_6) (99.9%), D_2O, and 2-mercaptoethanol (98%) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Acetonitrile and water (HPLC grade) were purchased from Baker Chemical Co. (Phillipsburg, NJ, USA). Silica gel 60 (70–230 mesh) was purchased from Merck Chemical. Co. (Darmstadt, Germany). Ammonia water (25–30%), n-propanol (99%), H_2O_2 (28%), and methylene chloride (99%) were purchased from Duksan Pure Chemical Co. (Ansan, GyonggiDo, Korea). A Whatman GF/C glass microfiber filter membrane (Whatman, UK) with a pore size of 1.2 μm and an Advantec PTFE filter membrane (Advantec, Japan) with a pore size of 0.2 μm were used for filtration.

2.2. Preparation of DBDY

For the large-scale synthesis of DBDY, 1 L of 5 mM BY solution was prepared in 0.1 M borate buffer (pH 9.1). HRP was added to an activity of 2260 purpurogallin units/L. Oxidation was initiated by adding H_2O_2 to a concentration of 2.5 mM. After 20 min, the reaction was terminated by adding 2-mercaptoethanol to a concentration of 5 mM. For comparison, DY was directly prepared from Y using HRP under the same conditions. All the reactions were performed at 39 °C [22].

To minimize the loading volume in silica column chromatography, DBDY was concentrated 100 times by evaporating under vacuum at 45 °C. Evaporation at a temperature higher than 50 °C was found to result in the decomposition of the Boc group (data not shown). Precipitated buffer components were removed by filtration through a Whatman GF/C glass microfiber filter membrane. The concentrated solution was stored at 4 °C prior to purification. Additional precipitates formed during storage were also removed by filtration.

2.3. Purification of DBDY by one-step silica column chromatography

The apparatus for silica column chromatography is shown in Fig. 1. Dry silica gel 60 was packed into a stainless steel column (150 cm × 5 cm i.d.) that was plugged with a stainless steel net and sea sand (20–30 mesh; Junsei, Japan) at both ends. Deionized water was first passed through the silica column to remove any trapped air. After equilibration with an eluent composed of n-propanol and ammonia:water (90:10, v/v), the column was connected to an injector, which consisted of a glass column with two screw caps (30 cm × 2 cm i.d.). Concentrated reaction solution (5–10 ml) was added to the injector. As soon as the sample was loaded onto the silica column, the injector was filled with the eluent. Passage of vaporized ammonia caused a crack in the packed silica gel; therefore, a diaphragm pump (model no. 07090-62; Masterflex AG, Gelsenkirchen, Germany) was used to pass the eluent through the column. Separation was performed at a flow rate of 60 ml/min and at a temperature below 15 °C to minimize the volatilization of ammonia. HPLC analysis of the elution fractions revealed that DBDY had a retention time of approximately 90 min. The fractions containing DBDY were collected and concentrated by vacuum evaporation at 45 °C.

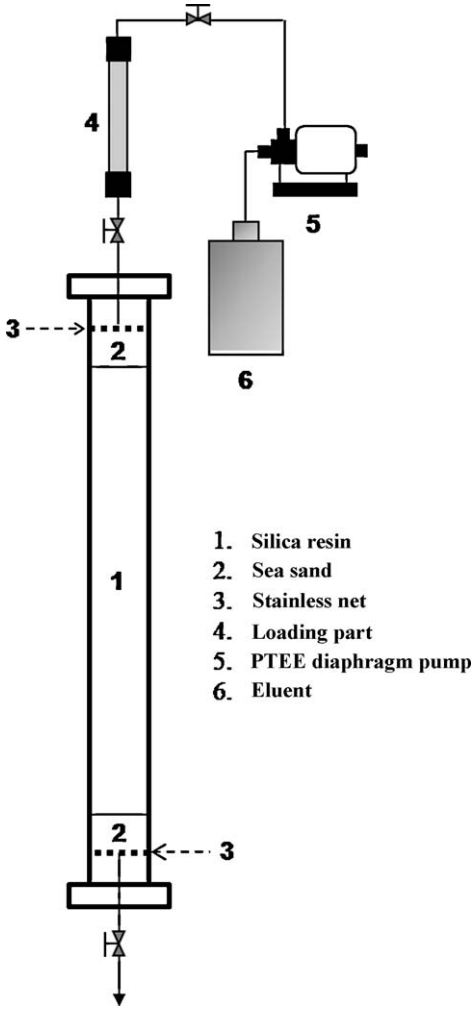

Fig. 1. The apparatus used for silica column chromatography. Dry silica gel 60 was packed into a stainless steel column (150 cm × 5 cm i.d.) that was plugged with a stainless steel net and sea sand (20–30 mesh; Junsei, Japan) at both ends. Deionized water was first passed through the silica column to remove any trapped air. After equilibration with an eluent composed of n-propanol and ammonia:water (90:10, v/v), the column was connected to an injector, which consisted of a glass column with two screw caps (30 cm × 2 cm i.d.).

2.4. Removal of the Boc groups in DBDY

To remove the Boc groups, 0.5 g of purified DBDY was dissolved in 20 ml methylene chloride. The deblocking reaction was initiated by adding 20 ml TFA [24–27]. After 30 min, the solution was evaporated under vacuum at 45 °C. The residue was then dissolved in deionized water, and undissolved materials were removed by filtration through an 0.2 μm PTFE filter membrane. Deionized water was removed by evaporation under vacuum at 45 °C. The residue was dissolved in DMF and finally reprecipitated in an excess of methylene chloride. The precipitate (DY) was collected by filtration through a PTFE membrane of pore size 0.2 μm.

2.5. Analysis

The products of the HRP-catalyzed oxidation reaction and fractions from the silica chromatography column were analyzed by using a HPLC system (Waters 510, Waters Corp., USA) equipped with a UV absorbance detector (Waters 486, Waters Corp., USA) [2] and a mass spectrometer (Triple Quadrupole Tandem Mass Spectrometer, Micromass and Waters, USA). Separation was carried out on a C-18 column (5 μm particle size, 250 mm × 4.6 mm, 80 Å pore size, Phenomenex, USA) using a mixture of 0.1% (v/v) trifluoroacetic acid solution and acetonitrile as the eluent. For

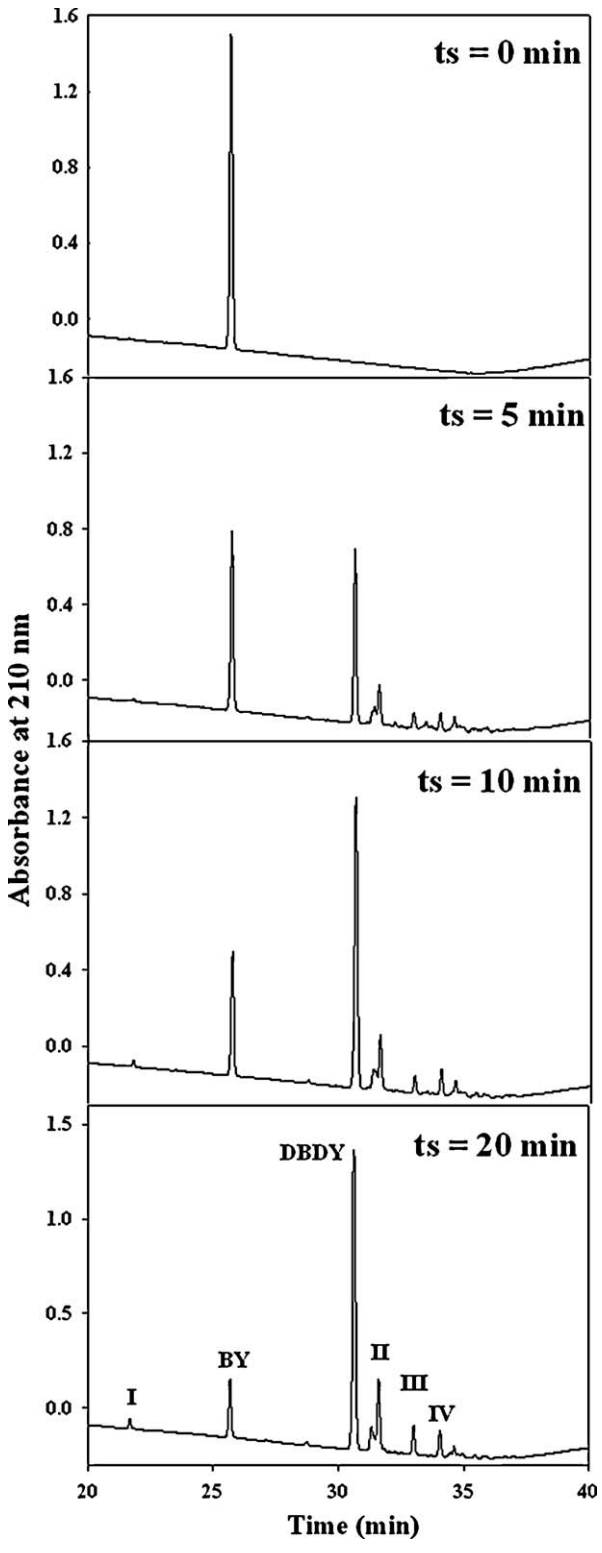

Fig. 2. HPLC chromatograms of oxidation products sampled at different reaction times. The time of sampling is denoted by "ts.". For the synthesis of DBDY, 5 mM BY solution was prepared in 0.1 M borate buffer (pH 9.1). HRP was added to an activity of 2260 purpurogallin units/L. Oxidation was initiated by adding H_2O_2 to a concentration of 2.5 mM. After 20 min, the reaction was terminated by adding 2-mercaptoethanol to a concentration of 5 mM. The reaction temperature was maintained at 39 °C. The HPLC separation was carried out on a C-18 column (5 μm particle size, 250 mm × 4.6 mm, 80 Å pore size, Phenomenex, USA) using a mixture of 0.1% (v/v) trifluoroacetic acid solution and acetonitrile as the eluent. Elution was carried out with a linearly increasing gradient of acetonitrile (from 0% to 60%) in 30 min. The flow rate of the eluent was maintained at 1 ml/min.

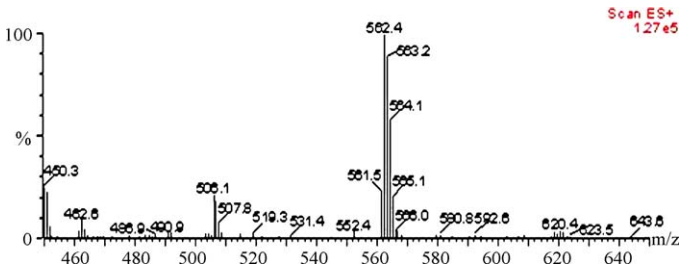

Fig. 3. Mass spectrum of DBDY obtained using a mass spectrometer (Triple Quadrupole Tandem Mass Spectrometer, Micromass and Waters, USA).

better separation of the oxidation products, elution was carried out with a linearly increasing gradient of acetonitrile (from 0% to 60%) in 30 min. The flow rate of the eluent was maintained at 1 ml/min.

The structures of DBDY and DY were identified based on their ^{1}H NMR spectra that were recorded on a Bruker Advance 500-MHz nuclear magnetic resonance spectrometer (Bruker BioSpin Corp., Germany). Prior to analysis, DBDY and DY were dissolved in D_2O. The ^{1}H NMR spectrum of DBDY was compared with that of DMF to determine the purity of purified DBDY. DBDY and DMF were dissolved in DMSO for this analysis. The DBDY purity was calculated using the ratio of the peak area of the aromatic ring protons in DBDY to that of methyl protons in DMF.

3. Results and discussion

3.1. Characterization of the oxidation products

HRP-catalyzed oxidation of BY was monitored by HPLC, and the result is shown in Fig. 2. The compounds present in the six major peaks had retention times (RT) of 21.7, 25.7, 30.6, 31.6, 33.0, and

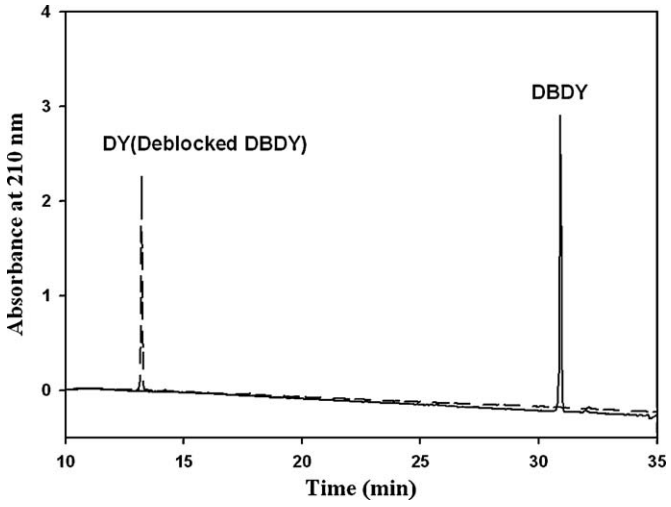

Fig. 4. The HPLC chromatograms of DBDY (—) and DY (---). DBDY was purified from the products of BY oxidation by one-step silica column chromatography. DY was then prepared by the removal of Boc groups in DBDY. The apparatus used for silica column chromatography is shown in Fig. 1. The purification of DBDY was initiated by adding the concentrated reaction solution (5–10 ml) to the injector. As soon as the sample was loaded onto the silica column, the injector was filled with the eluent. To avoid the passage of vaporized ammonia, a diaphragm pump (model no. 07090-62; Masterflex AG, Gelsenkirchen, Germany) was used to pass the eluent through the column. Separation was performed at a flow rate of 60 ml/min and at a temperature below 15 °C to minimize the volatilization of ammonia. HPLC analysis of the elution fractions revealed that DBDY had a retention time of approximately 90 min. The fractions containing DBDY were collected and concentrated by vacuum evaporation at 45 °C. The condition of the HPLC separation is described in the legend for Fig. 2. For the removal of Boc groups, purified DBDY was trated with TFA for 30 min. After vacuum evaporation at 45 °C, the residue was dissolved in deionized water, and undissolved materials were removed by filtration through an 0.2 μm PTFE filter membrane. Deionized water was removed by evaporation under vacuum at 45 °C. The residue was dissolved in DMF and finally reprecipitated in an excess of methylene chloride. The precipitate (DY) was collected by filtration through a PTFE membrane of pore size 0.2 μm.

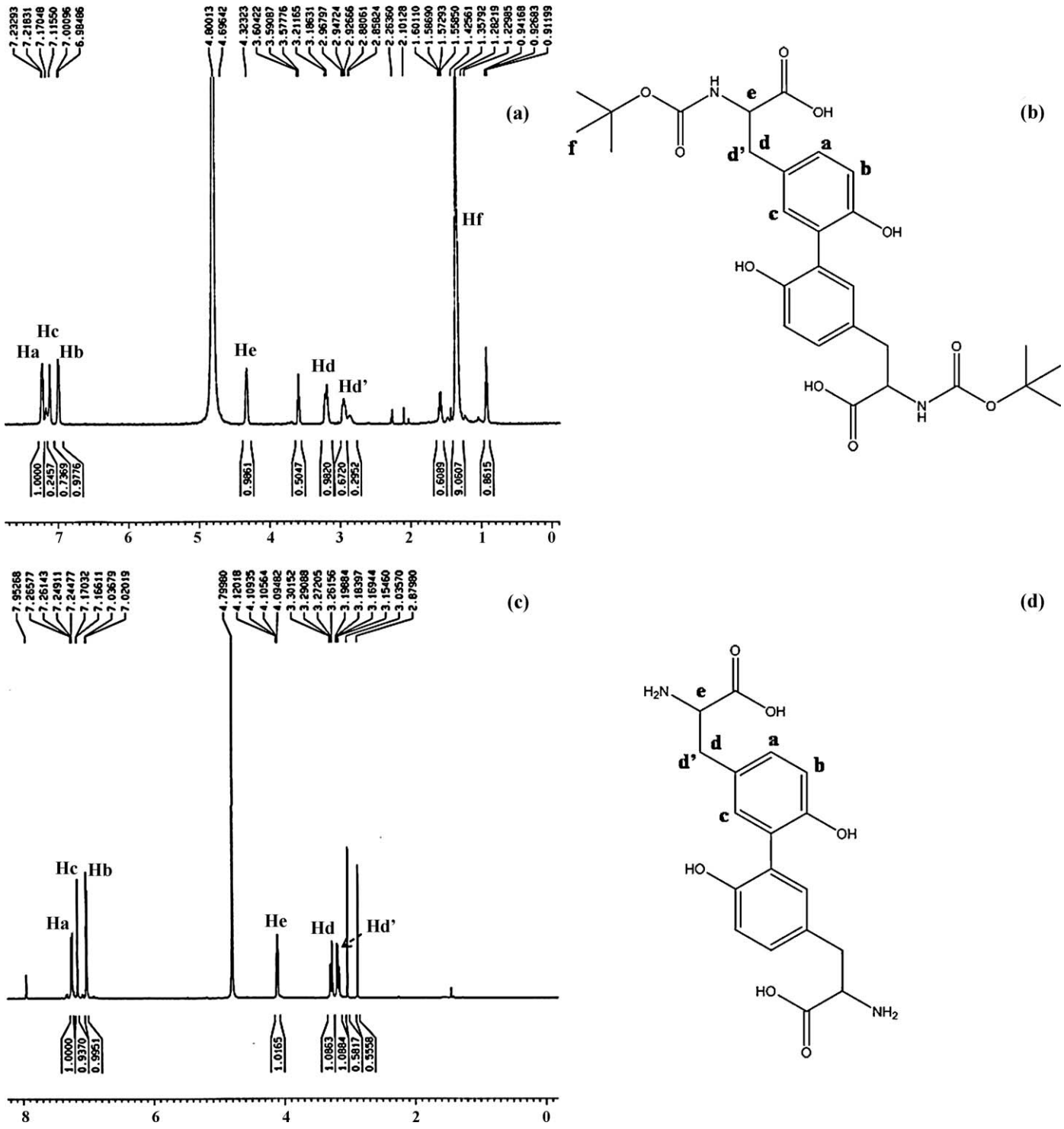

Fig. 5. ^{1}H NMR spectra of DBDY (A) and DY (C) obtained after the removal of Boc groups in DBDY. These spectra were recorded on a Bruker Advance 500-MHz nuclear magnetic resonance spectrometer (Bruker BioSpin Corp., Germany). Both compounds were dissolved in D_2O. The chemical structures of DBDY and DY are shown in (B) and (D), respectively.

34.0 min, respectively. These were then analyzed by mass spectroscopy (positive mode detection). The compound with an RT of 31.4 min disappeared during the concentration step, and its mass spectrum could not be obtained. The compound in the tiny peak with an RT of 21.7 min, which was labeled as I in Fig. 2, showed a mass value (m/z) of 462.2, indicating a molecular mass of 461 Da. The data indicated that this compound is *N*-Boc-dityrosine, and it is believed to be formed by the accidental loss of one Boc group from DBDY. The compound with an RT of 25.7 min showed a mass value (m/z) of 283.2, indicating that it is residual BY of molecular weight 282 Da. A fluorescent compound in the largest peak with RT of 30.6 min showed a mass value (m/z) of 562 (see Fig. 3), indicating that it is DBDY. The compound with an RT of 31.6 min, which was labeled as II in Fig. 2, also showed a mass value (m/z) of 562. Its mass spectrum (data not shown) indicated that it is another dimer of BY, i.e., *N*,*N*′-diBoc-isodityrosine. Compounds with RT values of 33.0 and 34.0 min showed a mass value (m/z) of 841, indicating that they are trimers of BY such as *N*,*N*′,*N*″-triBoc-trityrosine and

Table 1
Peak areas of BY, DBDY, Y, and DY in the HPLC chromatograms of samples that were taken during HRP-catalyzed oxidation of BY and Y. Detailed conditions of the reaction and the HPLC separation are described in the legend for Fig. 2.

Reaction time (min)	BY	DBDY	Reaction time (min)	L-Tyrosine	DY
0.0	13259.8	0.0	0.0	9421.7	0.0
5.0	6808.7	6433.2	40	4005.5	3100.8
10.0	4617.3	12595.3	80	3802.9	4975.1
20.0	2148.0	14206.2	120	2770.0	4985.4

N,*N*′,*N*″-triBoc-pulcherosine (see the peaks labeled as III and IV in Fig. 2).

3.2. Accumulation of DBDY during HRP-catalyzed oxidation of BY

The peak areas of BY, DBDY, Y, and DY in the HPLC chromatograms (see Fig. 2) are summarized in Table 1. Approximately 5 min was required for 50% reaction of BY, but approximately 40 min was needed for Y. In the last two samples, the increase in the peak area of DBDY was coupled with a decrease in the peak area of BY. However, the peak area of DY did not change even though the peak area of Y clearly decreased. This indicates that the conversion of Y to DY and the subsequent oxidation of DY occurred simultaneously during this time period. Further oxidation such as this was much lower in the case of DBDY, leading to a continuous increase in its peak area. Amado et al. [18] reported similar results with AY.

Michon et al. [28] recently reported the kinetics of the HRP-catalyzed oxidation of Y, AY, *N*-acetyltyrosine amide (AYA), and tyrosine-containing peptides. They found that the dimerization of AY and AYA (i.e., the conversion of AY and AYA to DADY and *N*,*N*′-diacetyl-dityrosine amide) was about 20 times faster than that of Y (i.e., the conversion of Y to DY). They suggested that the dimerization rate might depend on the positive ionization of the α-amino group because AY and AYA were devoid of a free amino group. They also found that the polymerization of AY and tyrosine-containing peptides (i.e., the subsequent oxidation of their dimers) was less than that of Y. Steric hindrance was suggested to be a reason for this result [28]. Their study of HRP kinetics also demonstrated that the polymerization was hindered due to the enzyme inhibition by the dimers of AY and tyrosine-containing peptides. Hence, the faster conversion of BY to DBDY than Y to DY can be explained by the absence of a free amino group in BY. The subsequent oxidation of DBDY is believed to have been hindered due to steric hindrance and the inhibition of HRP by DBDY, which resulted in the accumulation of DBDY.

3.3. Purification of DBDY by silica column chromatography

In silica column chromatography, the products of BY oxidation were eluted in the following order: BY, *N*,*N*′-diBoc-isodityrosine, DBDY, and trimers of BY. Once the time interval of DBDY elution was determined, the fractions during that interval were collected. The HPLC chromatogram of the collected fractions is shown in Fig. 4. It confirms that the DBDY obtained was highly pure. Fig. 5A shows the ^{1}H NMR spectrum of DBDY purified when loading 5 ml of the concentrated reaction solution to the silica column. From the comparison with the ^{1}H NMR spectrum of DMF (data not shown), the yield and the purity of DBDY were determined to be 37.5% and 89.7%, respectively; these values were comparable to those reported for DY (>26% yield and 91.9% purity) [22].

3.4. Removal of Boc groups from DBDY

It has been reported that *tert*-butyl cation and *tert*-butyl trifluoroacetate produced in the deblocking reaction might lead to alkylation at the *ortho* position of the hydroxyl group in tyrosine [29,30]. Therefore, 1,2-ethanedithiol was added to inhibit such an alkylation reaction, if any. However, the alkylation reaction did not occur, and all DBDY was converted to DY (see Fig. 4), irrespective of whether or not 1,2-ethanedithiol was added.

The ^{1}H NMR spectrum of DY, which was obtained by deblocking of DBDY, is shown in Fig. 5C. A singlet for methyl protons (1.36 ppm) designated as Hf (see Fig. 5A and B) is observed in Fig. 5A but is not seen in Fig. 5C, which proves that the Boc groups were completely removed (compare Fig. 5B and D). Moreover, the spectrum shown in Fig. 5C is identical to the ^{1}H NMR spectrum of DY reported elsewhere [19–21].

4. Conclusions

In this study, HRP-catalyzed oxidation of *N*-Boc-L-tyrosine followed by one-step silica column chromatography was presented as a method for the simple and large-scale preparation of dityrosine (DY). The presence of the *tert*-butoxycarbonyl (Boc) group allowed faster production of *N*,*N*′-diBoc-dityrosine (DBDY), the target product of the HRP-catalyzed oxidation reaction, and a higher yield. The yield and purity of DBDY were up to 37.5% and 89.7%, respectively. Reaction with trifluoroacetic acid permitted the easy removal of Boc groups in DBDY, and all of the purified DBDY was completely converted to DY. DBDY is considered to be better than DY for subsequent chemical reactions (for binding to a polymer, amino acid, drug, antibody, etc.) because these reactions can be selectively performed by using the carboxylic acid and amine groups in the following sequence: first, the carboxylic acid groups are used; next, the Boc groups are removed; and finally, the amino groups are used.

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References

[1] Hensley K, Maidt ML, Yu Z, Sang H, Markesbury WR, Floyd RA. Electrochemical analysis of protein nitrotyrosine and dityrosine in the Alzheimer brain indicates region-specific accumulation. J Neurosci 1998;18:8126–32.
[2] Souza JM, Giasson BI, Chen Q, Lee VM-Y, Ischiropoulos H. Dityrosine cross-linking promotes formation of stable α-synuclein polymers. J Biol Chem 2000;275:18344–9.
[3] Smith DG, Cappai R, Barnham KJ. The redox chemistry of the Alzheimer's disease amyloid β peptide. Biochim Biophys Acta 2007;1768:1976–90.
[4] van der Vliet A, Nguyen MN, Shigenaga MK, Eiserich JP, Marelich GP, Cross CE. Myeloperoxidase and protein oxidation in cystic fibrosis. Am J Physiol Lung Cell Mol Physiol 2000;279:L537–46.
[5] Jacob JS, Cistola DP, Hsu FF, Muzaffar S, Mueller DM, Hazen SL, et al. Human phagocytes employ the myeloperoxidase-hydrogen peroxide system to synthesize dityrosine, trityrosine, pulcherosine, and isodityrosine by a tyrosyl radical-dependent pathway. J Biol Chem 1996;271:19950–6.
[6] Yoburn JC, Tian W, Brower JO, Nowick JS, Glabe CG, Van Vranken DL. Dityrosine cross-linked Ab peptides: fibrillar b-Structure in Ab(1–40) is conductive to formation of dityrosine cross-links but a dityrosine cross-link in Ab(1–40) does not induce b-structure. Chem Res Toxicol 2003;16:531–5.
[7] Giulivi C, Davies KJA. Dityrosine: a marker for oxidatively modified proteins and selective proteolysis. Methods Enzymol 1994;233:363–71.
[8] Giulivi C, Davies KJA. Mechanism of the formation and proteolytic release or H_2O_2-induced dityrosine oxidation products in hemoglobin and red blood cells. J Biol Chem 2001;276:24129–36.

[9] Dimarco T, Giulivi C. Current analytical methods for the detection of dityrosine, a biomarker of oxidative stress, in biological samples. Mass Spectrom Rev 2007;26:108–20.
[10] Malencik DA, Anderson SR. Dityrosine as a product of oxidative stress and fluorescent probe. Amino Acids 2003;25:233–47.
[11] Briza P, Winkler G, Kalchhauser H, Breitenbach M. Dityrosine is a prominent component of the yeast ascospore wall. A proof of its structure. J Biol Chem 1986;261:4288–94.
[12] Li J, Hodgeman BA, Christensen BM. Involvement of peroxidase in chorion hardening in (*Aedes aegypti*). Insect Biochem Mol Biol 1996;26:309–17.
[13] Nomura K, Suzuki N. Sea urchin ovoperoxidase: solubilization and isolation from the fertilization envelope, some structural and functional properties, and degradation by hatching enzyme. Arch Biochem Biophys 1995;319:525–34.
[14] LaBella F, Waykole P, Queen G. Formation of insoluble gels and dityrosine by the action of peroxidase on soluble collagens. Biochem Biophys Res Commun 1968;30:333–8.
[15] Tilley M, Benjamin RE, Srivarin P, Tilley KA. Nonenzymatic preparative-scale synthesis of dityrosine and 3-bromotyrosine. Anal Biochem 2004;334:193–5.
[16] Breitenkamp K, Sill K, Skaff H, Breintenkamp R. Polymeric micelles for drug delivery. WO 06107903; 2006.
[17] Fry SC. Isodityrosine, a diphenyl ether cross-link in plant cell wall glycoprotein: identification, assay, and chemical synthesis. Methods Enzymol 1984;107:388–97.
[18] Amado R, Aeschbach R, Neukom H. Dityrosine: *in vitro* production and characterization. Methods Enzymol 1984;107:377–88.
[19] Nishiyama S, Kim MH, Yamamura S. Syntheses of isodityrosine, dityrosine and related componds by phenolic oxidation of tyrosine and phenylglycine derivatives using an electrochemical method. Tetrahedron Lett 1994;35:8397–400.
[20] Hutton CA, Skaff O. A convenient preparation of dityrosine via Miyaura borylation–Suzuki coupling of iodotyrosine derivatives. Tetrahedron Lett 2003;44:4895–8.
[21] Lee DI, Hwang SP, Choi JY, Ahn IS, Lee CH. A convenient preparation of dityrosine via Mn(III)-mediated oxidation of tyrosine. Process Biochem 2008;43:999–1003.
[22] Malencik DA, Sprouse JF, Swanson CA, Anderson SR. Dityrosine: preparation, isolation, and analysis. Anal Biochem 1996;242:202–13.
[23] Bodanszky M. Principles of peptide synthesis. 2nd ed. Berlin/Heidelberg: Springer-Verlag; 1993. p. 65–74.
[24] Agami C, Couty F. The reactivity of the *N*-Boc protecting group: an underrated feature. Tetrahedron 2002;58:2701–24.
[25] Ahn KD, Koo DI. Synthesis and polymerization of t-Boc protected maleimide monomers: N-(t-butyloxycarbonyloxy)maleimide and N-[p-(t-butyloxycarbonyloxy)phenyl]maleimide. Polymer 1995;36:2621–8.
[26] Strazzolini P, Melloni T, Giumanini AG. Selective nitrolytic deprotection of *N*-Boc-amines and *N*-Boc-amino acids derivatives. Tetrahedron 2001;57:9033–43.
[27] Dandepally SR, Williams AL. Microwave-assisted N-Boc deprotection under mild basic conditions using $K_3PO_4{\cdot}H_2O$ in MeOH. Tetrahedron Lett 2009;50:1071–4.
[28] Michon T, Chenu M, Kellershon N, Desmadril M, Gueguen J. Horseradish peroxidase oxidation of tyrosine-containing peptides and their subsequent polymerization: a kinetic study. Biochemistry 1997;36:8504–13.
[29] Lundt BF, Johansen NL, Volund A, Markussen J. Removal of t-butyl and t-butoxycarbonyl protecting groups with trifluoroacetic acid. Int J Pept Protein Res 1978;12:258–68.
[30] Benoiton NL. Chemistry of peptide synthesis. CRC Press; 2006. p. 71–73.