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Enhancing the water dispersibility of paclitaxel by complexation with hydrophobic peptides

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ABSTRACT

The complex between paclitaxel (Ptx) and a peptide mixture (Pep) was prepared to enhance of the water-dispersibility of Ptx. Pep was prepared by enzymatic hydrolysis of casein, followed by fractionation using ammonium sulfate precipitation and ultrafiltration. The Ptx and Pep complex (Ptx–Pep) was prepared by mixing an ethanol solution of Ptx and an aqueous solution of Pep followed by lyophilization. The water dispersibility test of Ptx–Pep prepared using different fractions of Pep demonstrated that a fraction (Pep-A), containing relatively hydrophobic peptides with high molecular weights, was effective in enhancing the water dispersibility of Ptx. The sequences of the major peptides in Pep-A were identified by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry with "LIFT" technique. The water dispersibility of the complex between Ptx and Pep-A (Ptx–Pep-A) was independent of pH, even though it is positively or negatively charged under strongly acidic and neutral conditions. As the particle size of Ptx–Pep-A in aqueous media was 147–215 nm, Ptx–Pep-A was present as a hydrocolloidal material in aqueous media.

1. Introduction

Paclitaxel (Ptx), which is a diterpenoid isolated from the bark of Pacific Yew (*Taxus Brevifolia*), is an effective anticancer drug that demonstrates activity against a wide range of tumors, such as head and neck, ovarian, breast, and non-small-cell lung cancers [1–5]. The major obstacle for the use of Ptx is its low water solubility (0.7 mg/L), which is attributed to high hydrophobicity [6]. Therefore, Ptx is currently formulated as Taxol® which includes a mixture of polyoxyethylated castor oil (Cremophor® EL) and dehydrated ethanol (1:1, v/v). However, when administered by intravenous infusion, Cremophor® EL causes serious side effects [7], such as acute hypersensitivity reactions in some patients.

Various techniques for the aqueous-based formulation of Ptx that are devoid of Cremophor EL® have been investigated [8,9]. Various amphiphilic block copolymers have been developed as self-assembled nanocarriers for Ptx. Biodegradable multiblock poly(*N*-2-hydroxypropyl) methacrylamide copolymer, gemcitabine and Ptx conjugates were developed as a nanocarrier for the treatment of ovarian cancer [10]. Ptx-loaded poly(lactic-co-glycolic acid) nanometric (<200 nm) spherical nanoparticles have also been prepared [11]. Transferrin conjugated poly (γ-glutamic acid-maleimide-co-l-lactide)-1,2-dipalmitoylsn-glycero-3-phosphoethanolamine copolymer was developed as a nanoparticle of Ptx [12]. Poly(ethylene glycol)-β-poly(ϵ-caprolactone) was prepared for the micellar formulation of a Ptx prodrug [13]. Recently, a linear–dendritic copolymer composed of polyethylene glycol and all-trans-retinoic acid was prepared as drug delivery platform for Ptx [14]. Liposomes have also been developed as advanced vehicles for Ptx [15–17]. Ptx can be entrapped in the liposomal phospholipid bilayers, resulting in enhanced therapeutic efficacy, and a liposomal Ptx formulation (Lipusu®) has been commercialized. Emulsification has been studied as the formulation technique for Ptx [18,19]. A supersaturable self-emulsifying

Abbreviations: Ptx, paclitaxel; Pep, peptide mixture prepared as casein hydrolysate; Ptx–Pep, the complex between paclitaxel (Ptx) and Pep; Pep-A, the Pep fraction obtained from a combination of 19 wt% $(NH4)2SO4$ precipitation and an ultrafiltration membrane with a molecular weight cutoff of 5000 gmol−1; Ptx–Pep-A, the complex between Ptx and Pep-A; MALDI LIFT-TOF/TOF MS, matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometer with "LIFT" technique; PBS, phosphate-buffered solution; Ptx-Blank, a blank sample (for Ptx-Blank) prepared by mixing an aqueous solution without Pep-A and an ethanol solution containing Ptx; F1, centrifuged sample (fraction 1); F2, filtrate through a 0.80 μm membrane filter (fraction 2); F3, filtrate through a 0.45 μm membrane filter (fraction 3); F4, filtrate through a 0.20 μm membrane filter (fraction 4); F5, filtrate through a molecular weight cutoff of 200,000 gmol−1 (fraction 5); GRAVY, the grand average of hydropathicity; β-CN, β-casein; α_{S1}-CN, α_{S1}-casein; $Conc./Conc._{F1}$, the relative concentration of Ptx in fractions F2–F4 compared with F1.

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drug delivery system of Ptx has been developed by employing hydroxypropyl methylcellulose as a precipitation inhibitor [20]. Cyclic oligosaccharides cyclodextrins have also been demonstrated as solubilizing agents for Ptx [21–24]. A series of cyclodextrins form inclusion complexes with Ptx to enhance the water solubility.

Conjugation with human serum albumin is found to be effective at enhancing the therapeutic efficacy of Ptx. The conjugates prepared by covalently attaching Ptx to human serum albumin are stable and show linear Ptx release in the presence of proteases or liver extracts *in vitro* and *in vivo* with high cytotoxicity [25]. Abraxane®, albumin-bound Ptx nano particles, has been approved by the US FDA for the treatment of metastatic breast cancer and non-small-cell lung cancer [26,27]. Noncovalent binding between Ptx and human serum albumin was also investigated as a water soluble formulation [28]. Such complex formation with biomolecules would enhance the hydrophilicity of Ptx [29].

Recently, a peptide mixture (abbreviated as Pep), which was obtained as casein hydrolysate, has been developed as an excipient for poorly water-soluble drugs and nutraceuticals to enhance the water solubility or water dispersibility [30]. Poorly water-soluble drug indomethacin was mixed with Pep to obtain a complex which was much more water-soluble than that of indomethacin alone under weakly acidic and neutral conditions [30,31]. The resulting complex is quite small and permeable through ultrafiltration membranes. Similarly, complexation of coenzyme Q_{10} with albumin hydrolysate enhances the water dispersibility of coenzyme Q_{10} [32]. The complex was present as a hydrocolloid and the particle size in aqueous media was 170–280 nm. Pep contains various peptides with versatile sequences and peptide lengths, and part of the peptides should exhibit affinity to poorly water-soluble molecules based on various interactions, *e.g.*, hydrophobic, electrostatic. The complex between heme iron and peptide fragments (heme iron preparation, HIP) is commercially used as a supplement to prevent anemia [33,34]. HIP is apparently soluble in aqueous media and is more readily absorbed by the human body than heme iron alone [35].

As discussed above, complexation with Pep enhances the water solubility and/or water dispersibility for poorly water soluble molecules. However, Pep has been used as a mixture of protein hydrolysate, and the dominant peptides that interact with poorly water soluble molecules have not yet been specified. In the present study, the water dispersibility of the complex between Ptx and Pep (Ptx–Pep) was evaluated. In order to find effective peptides that interact with Ptx, Pep was fractionated by precipitation using ammonium sulfate solution, followed by ultrafiltration. Ptx–Pep was prepared by mixing an ethanol solution of Ptx and an aqueous solution of each fraction of Pep, followed by removal of ethanol *in vacuo* and lyophilization. The water dispersibilities of the Ptx–Pep complexes were compared to identify the most effective peptide fraction. Additionally, the molecular weight and sequence of part of the peptides in each fraction were identified using matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometer with "LIFT" technique (MALDI LIFT-TOF/TOF MS) [36]. Ptx–Pep prepared using the most effective fraction was assessed in subsequent dispersibility tests and characterized in particle size analysis and zeta potential in aqueous media. The morphology of Ptx–Pep was also characterized using scanning electron microscopy (SEM).

2. Material and methods

2.1. Materials

Ptx, milk casein, high-performance liquid chromatography (HPLC) grade acetonitrile (ACN), trifluoroacetic acid (TFA), polyvinylpyrrolidone K25 (PVP), polyvinyl alcohol 2000 (PVA), dextran 40,000 (Dex), gelatin from bovine bone (Gel), polyethylene glycol 500,000 (PEG 500000), and polyethylene glycol 4000 (PEG 4000) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). α-Chymotrypsin from bovine pancreas, α-cyano-4-hydroxycinnamic acid (CHCA), and poly(D,L-lactide-co-glycolide), acid terminated, (65:35) average Mw 24,000–38,000, RESOMER® RG 653H (PLGA) were purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan). All other reagents were analytical grade. Cellulose acetate disposable membrane filters (DISMIC®, pore size: 0.80 μm (25CS080AN), 0.45 μm (25CS045AN), and 0.20 μm (25CS020AN)), an ultrafiltration membrane made from polysulfone with molecular-weight cutoff points of 20,000 $gmol^{-1}$ (P0200 076E), and a polysulfone ultrafiltration unit with molecular-weight cutoff points of 200,000 $gmol^{-1}$ (USY-20) were purchased from Advantec Toyo (Tokyo, Japan). Ultrafiltration membranes (Ultracel® 5 kDa, Ultracel® 3 kDa, and Ultracel® 1 kDa) with molecular-weight cutoff points 5000, 3000, and 1000 $gmol^{-1}$, which are made of regenerated cellulose were purchased from Merck Millipore (Billerica, MA, USA) and used for fractionation of the peptides.

2.2. Preparation of Pep

Pep was obtained as a peptide mixture by enzymatic hydrolysis of casein using α-chymotrypsin in a similar manner as described previously [30,32]. Casein (50 g) was dissolved in 1000 mL of Milli-Q water and the pH was adjusted to 7.8 using sodium hydroxide. Calcium chloride dehydrate (3.0 g) was added to the solution and the temperature was adjusted to 45 °C. α-Chymotrypsin (250 mg) was added to the solution to hydrolyze the casein. After 6 h, the temperature was increased to 80 °C to inactivate the enzyme. After cooling, the solution supernatant was ultrafiltered using the ultrafiltration membrane with a molecular weight cutoff of 20,000 $gmol^{-1}$. The permeate was subsequently ultrafiltered using the ultrafiltration membrane with a molecular weight cutoff of 1000 $gmol^{-1}$. The retentate was lyophilized and a white powder was obtained as Pep. From the result of gel filtration chromatography HPLC analysis, Pep had a wide molecular-weight distribution. The major components were found to be 1100, 2600, 7100, and 10,500 $gmol^{-1}$ [30].

2.3. Fractionation of Pep by the combination of ammonium sulfate and ultrafiltration

Pep, which was prepared as described in Section 2.2, was fractionated by a stepwise ammonium sulfate precipitation, followed by ultrafiltration using ultrafilter membranes with different molecular-weight cutoffs (Fig. 1). Specifically, Pep (22.5 g) was dissolved in 450 mL of Milli-Q water. The pH of the solution was adjusted to 7.0 using small quantities of 6 M hydrochloric acid. The solution was kept below 5 °C in an ice-water bath for 30 min with stirring, then centrifuged at 4 °C, 10,000 × *g* for 10 min. Subsequently, 51.3 g (10 wt%) of ammonium sulfate was added to the supernatant. As the quantity of the precipitate was quite small, it was disposed. Successively, 19 wt% $(NH_4)_2SO_4$ was added to the resultant supernatant and the precipitate was recovered. In a similar manner, precipitates were obtained by adding 27 wt%, 34 wt%, and 40 wt% $(NH_4)_2SO_4$ in turn. Each precipitate obtained by ammonium sulfate precipitation was dissolved again by adding distilled water and the solution was ultrafiltered using an ultrafiltration unit (600 cm^3, UHP-90K, Advantec Toyo) using an ultrafiltration membrane with a molecular weight cutoff of 5000 $gmol^{-1}$ at 0.3 MPa until approximately 95 vol% of the filtrate had passed through the membrane. The retentate was lyophilized to obtain a fraction of Pep, and the permeate was successively ultrafiltered again using ultrafiltration membranes with molecular weight cutoffs of