Water Solubility of Complexes between a Peptide Mixture and Poorly Water-Soluble Ionic and Nonionic Drugs

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Recently, a peptide mixture (Pep) obtained as a casein hydrolysate was found to be effective for enhancing the water solubility or water dispersibility for poorly water-soluble drugs. In the present study, complexation of Pep with ionic and nonionic drugs indomethacin (Indo), ibuprofen (Ibu), and prednisolone (Pre) was studied. The water solubility of complexes containing Indo and Ibu, both of which have a dissociable carboxylic group, increased with increasing pH. In contrast, the water solubility of a complex containing Pre, which does not contain dissociable groups, was almost independent of pH. As all three complexes were permeable through an ultrafiltration membrane with a molecular-weight cutoff 10,000 gmol^{-1}, the complexes were present not as colloidal materials but relatively small species in aqueous media. Moreover, Indo, Ibu, and Pre were complexed with twelve peptide fractions, which were derived from Pep by combining ammonium sulfate precipitation with ultrafiltration. Water solubility of the drugs increased with all Pep-derived fractions, suggesting that various peptides interact with the drugs.

Introduction

A variety of active pharmaceutical ingredients have been developed through progress in high-throughput screening technology but over 40% of these are poorly water-soluble (Lipinski 2002; Vo, *et al.*, 2013). Over the past few decades, various solubilizing agents, such as cyclodextrins, phospholipids, surfactants, and water-soluble polymers, were developed to solubilize poorly water-soluble drugs (Strickley, 2004; Vasconcelos *et al.*, 2007; Hawkins *et al.*, 2008). Proteins have also been developed as vehicles for solubilizing poorly water-soluble drugs because of their relatively low-toxicity, biocompatibility and biodegradability (Yewale *et al.*, 2013; Lohcharoenkal *et al.* 2014). Folate-decorated docetaxel-loaded human serum albumin (HSA) nanoparticles and tamoxifen-loaded HSA nanoparticles with a particle size of approximately 200 nm were developed as carriers in drug delivery systems (DDSs) (Kouchakzadeh *et al.*, 2014; Jiang *et al.*, 2015). Abraxane®, which is an injectable suspension of albumin-bound paclitaxel (Ptx) particles, was developed and used for the treatment of advanced non-small-cell lung cancer (Green *et al.*, 2006). Beta-casein nanomicelles containing celecoxib were prepared, resulting in improved oral bioavailability (Perlstein *et al.*, 2014). Beta-casein was also used as a platform for oral delivery of Ptx (Shapira *et al.*, 2012). Nanoparticles of a vegetable protein, gliadin, derived from wheat gluten, were used as a drug carrier for the controlled release of all-*trans*-retinoic acid (Ezpeleta *et al.*, 1996). Moreover, zein, originating from maize seeds, was used as a tablet excipient for anhydrous theophylline (Georget *et al.*, 2008), and amino acids have been also studied as excipients. Proline was mixed with naproxen (Jensen *et al.*, 2014), while arginine, phenylalanine, tryptophan, and tyrosine were also mixed with indomethacin (Indo) or carbamazepine to form co-amorphous drugs with improved stability and dissolution (Löbmann *et al.* 2013a, 2013b). Amphiphilic copolymers poly(sodium *N* acryloyl-l-aminoacidate-cododecylacrylamide) (aminoacidate = glycinate, leucinate, or phenylalaninate) were synthesized to improve the solubility of griseofulvin as an application for DDSs (Dutta and Dey, 2011).

Recently, the authors developed peptide mixtures as novel excipients for poorly water-soluble ingredients (Inada *et al.*, 2013; Matsushita *et al.*, 2013; Oshima *et al.*, 2013). The water solubility of Indo was enhanced by complexation with a peptide mixture (abbreviated as Pep) prepared by enzymatic hydrolysis of milk casein. The resulting complex between Indo and Pep (Indo-Pep) was found to be small enough to pass thorough ultrafiltration membranes with a molecular-weight cutoff (MWCO) of 10,000 gmol^{-1}. Furthermore, the water dispersibility of coenzyme Q_{10} and Ptx was improved by complexation with protein hydrolysates. The complex between coenzyme Q_{10} and albumin hydrolysate was present as a hydrocolloidal material and the particle size in aqueous media was 170–280 nm (Inada *et al.* 2013; Matsushita *et al.* 2013; Oshima *et al.* 2013). The complex between Ptx and Pep was also present as a hydrocolloidal material and the particle size in aqueous media was 150–220 nm (Inada *et al.*, 2013; Matsushita *et al.*, 2013; Oshima *et al.*, 2013). These results indicate that complexes between poorly water-soluble ingredients and peptide mixtures would be water-soluble or water-dispersible (if not water-soluble). Additionally, the water solubility or water dispersibility of complexes with

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Indo and coenzyme Q_{10} depended on the pH (Inada *et al.*, 2013; Matsushita *et al.*, 2013; Oshima *et al.*, 2013), while the water dispersibility of complexes with Ptx was independent of the pH (Inada *et al.*, 2013; Matsushita *et al.*, 2013; Oshima *et al.*, 2013).

In a previous study, casein hydrolysate was used as an additive to enhance the dissolution rates and oral bioavailability of drugs (Kimura *et al.*, 1991). The dissolution rate of Ibu from a kneaded mixture with casein hydrolysate became higher than that of the drug alone. The dissolution behavior from a kneaded mixture of drugs (diclofenac acid, diazepam, and Pre) with two types of casein hydrolysate (mean peptide lengths 3.3 and 17.4) was also improved, depending on the peptide length of the casein fragments (Imai *et al.*, 1998). However, the conditions under which casein hydrolysate interact with a hydrophobic drug were not studied in detail. Complexes between drugs and Pep both in solution and in solid states should be analyzed for application as an excipient. Additionally, the interaction between a drug and casein hydrolysate would change whether the drug is ionic or nonionic. Furthermore, the dissolution state and the size of the complex are not clarified in previous studies.

In the present study, complexation of three types of poorly water-soluble drugs, Indo, ibuprofen (Ibu), and prednisolone (Pre), with Pep were studied (**Figure 1**). Indo and Ibu bear a carboxyl group, while Pre does not contain ionic groups. The water solubilities of these ionic and nonionic drugs after complexation with Pep were compared to study the effect of ionic groups on the water solubility. Additionally, the permeability using an ultrafiltration membrane and the particle sizes of the complexes were investigated, to confirm whether the complexes were water-soluble or water-dispersible. Moreover, Pep was fractionated by combining ammonium sulfate precipitation with ultrafiltration.

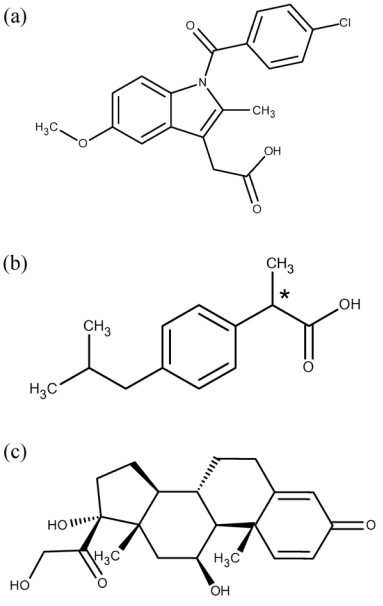

Fig. 1 Molecular structures of (a) indomethacin, (b) ibuprofen, and (c) prednisolone

The solubilities of complexes between the peptide fractions and drugs were compared to identify effective peptides that interact with drugs.

1. Materials and Methods

1.1 Materials

Indo, Ibu, Pre, milk casein, and high-performance liquid chromatography (HPLC) grade acetonitrile (ACN), glucose, sucrose, polyethylene glycol 4,000 (PEG 4,000), and polyethylene glycol 500,000 (PEG 500,000) were purchased from Wako Pure Chemical Industries, Ltd. α-Chymotrypsin from bovine pancreas was purchased from Sigma-Aldrich Japan K.K. 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 a molecular-weight cutoff (MWCO) of 20,000 $gmol^{-1}$ (P0200 076E), and a polysulfone ultrafiltration unit with a molecular-weight cutoff of 200,000 $gmol^{-1}$ (USY-20) were purchased from Advantec Toyo. Ultrafiltration membranes (Ultracel® 5 kDa, Ultracel® 3 kDa, and Ultracel® 1 kDa) with MWCOs of 5,000, 3,000, and 1,000 Da, respectively, which were made of regenerated cellulose were purchased from Merck Millipore Corp. and used for fractionation of the peptides.

1.2 Preparation of Pep and Pep fractions

Pep was obtained as a peptide mixture by enzymatic hydrolysis of casein using α-chymotrypsin, as follows (Inada *et al.*, 2013): casein (50 g) was dissolved in 500 cm^3 of Milli-Q water, and the pH was adjusted to 7.8 using sodium hydroxide. Calcium chloride dihydrate (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 casein. After 6 h, the mixture was heated at 80°C for 5 min to inactivate the enzyme. After cooling, the solution was successively ultrafiltered using UF membranes with a MWCO of 20,000 and 1,000 $gmol^{-1}$. Thereafter, the retentate was lyophilized and Pep was obtained as a white powder. From the results of gel filtration chromatography/high-performance liquid chromatography, Pep was shown to have a wide molecular-weight distribution, with major components at 1,100, 2,600, 7,100, and 10,500 $gmol^{-1}$ (Inada *et al.*, 2013).

Subsequently, Pep was fractionated by stepwise ammonium sulfate precipitation, followed by ultrafiltration using UF membranes with various MWCOs (**Figure 2**), to find effective peptides that interact with drugs (Inada *et al.*, 2015). Pep (22.5 g) was dissolved in 450 mL of Milli-Q water. The pH of the solution was adjusted to 7.0 using a small quantity 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 and 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 not used in this experiment. Subsequently, 19 wt% $(NH_4)_2SO_4$ was added to the resultant supernatant and the

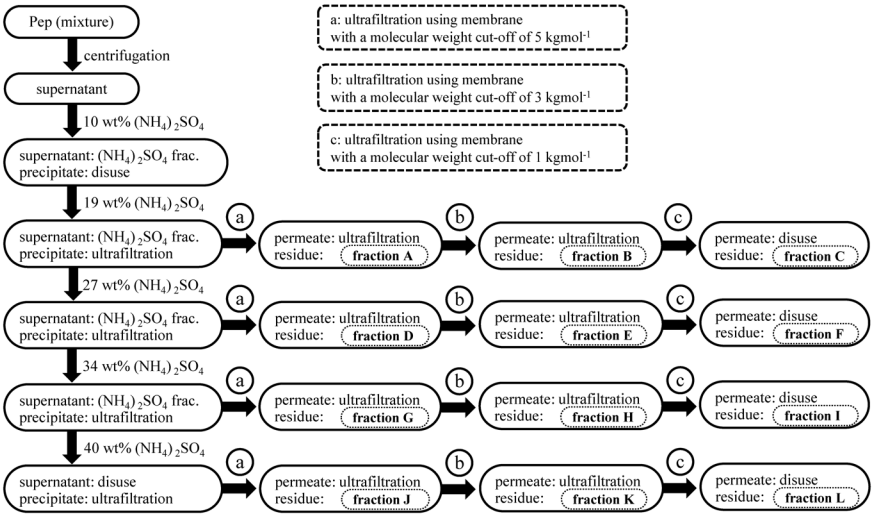

Fig. 2 Flowchart for the fractionation of Pep, combining ammonium sulfate precipitation and ultrafiltration

precipitate was recovered. In a similar manner, precipitates were obtained by adding 27, 34, and 40 wt% $(NH_4)_2SO_4$ in turn. Each precipitate was re-dissolved by adding distilled water and the solution was ultrafiltered using a UF membrane with a MWCO of 5,000 $gmol^{-1}$. The retentate was then lyophilized to obtain the Pep fraction, while the permeate was successively ultrafiltered again using UF membranes with MWCOs of 3,000 and 1,000 $gmol^{-1}$. The retentates from each membrane treatment were then lyophilized. Finally, twelve Pep fractions A–L containing different peptides were prepared.

1.3 Preparation of complexes of drugs and Pep and the water-solubility test

Table 1 shows the preparation conditions for complexes of three drugs with Pep. The complexes between drugs and Pep were prepared as follows: An ethanol solution of a drug and an aqueous solution of Pep were prepared. The solutions (2.5 mL each) were mixed and shaken at 30°C (120 rpm) for 1 h. After the ethanol was removed *in vacuo*, the residue was lyophilized to obtain the complexes as white powders. A blank sample was also prepared by mixing distilled water (2.5 mL) without Pep and an ethanol solution (2.5 mL) containing the drugs.

Table 2 shows conditions for the solubility test of complexes. To evaluate of the water solubility of the complexes, each sample was added to 5.0 mL of phosphate buffered solution (PBS; 100 mM). The aqueous mixture was then shaken at 30°C (120 rpm) for 1 h. After membrane filtration of the aqueous mixture, the pH of the filtrate was measured using a pH meter (HM-30G, DKK-TOA Co.). The concentration of the complexes was determined by UV-Vis spectrophotometer (V-660, JASCO) or reversed phase HPLC (Prominence gradient system, Shimadzu Corp.) on a C_{18} column (3.5 μm, 4.6 mm×150 mm, C18 XBridge BEH Waters) at 40°C. The mobile phase was a mixture of ACN and PBS (pH 2.5, 10 mM). The eluent was monitored by a Shimadzu SPD-20 A UV-Vis detector.

Table 1 Preparation conditions of the complexes with Indo, Ibu, and Pre

Preparation condition	Drug		
	Indo	Ibu	Pre
Concentration of drug ethanol solution [g/L]	2.0	10.0	1.0
Concentration of Pep aqueous solution [g/L]	20.0	20.0	20.0
Volume of drug ethanol solution [mL]	2.5	2.5	2.5
Volume of Pep aqueous solution [mL]	2.5	2.5	2.5
Shaking temperature [°C]	30	30	30
Shaking speed [rpm]	120	120	120
Shaking time [h]	1	1	1

Table 2 Solubility test and analysis conditions of the complexes with Indo, Ibu, and Pre used for pH dependence tests

Solubility test condition	Drug		
	Indo	Ibu	Pre
Volume of PBS (100 mM) [mL]	5.0	5.0	5.0
pH range of PBS (100 mM)	2.7–6.4	2.6–6.0	2.0–6.0
Shaking temperature [°C]	30	30	30
Shaking speed [rpm]	120	120	120
Shaking time [h]	1	1	1
Pore size of membrane filter [μm]	0.20	0.45	0.45
Analytical method	UV-Vis	HPLC	HPLC
Wavelength of UV-Vis detector [nm]	320	230	245
HPLC condition			
Column temperature [°C]	—	40	40
Mobile phase ratio of ACN/PBS (10 mM)	—	45/55	30/70
pH of mobile phase	—	2.5	2.5

Moreover, complexes between drugs and other excipients (glucose, sucrose, PEG 4,000, and PEG 500,000) were prepared by mixing ethanol solutions of the three drugs (Indo; 2.0 g/L, Ibu; 10.0 g/L, Pre; 1.0 g/L, 2.5 mL) and an aqueous solution of the excipients (20.0 g/L, 2.5 mL), followed by shaking at 30°C (120 rpm) for 1 h, and lyophilization. To evaluate the water solubility of the complexes, each sample

was added to 5.0 mL of PBS (100 mM). The aqueous mixture was shaken at 30°C (120 rpm) for 1 h. After membrane filtration of the aqueous mixture, the pH of the filtrate was measured using a pH meter, followed by determining the concentration of the complexes by UV-Vis (for Indo) and HPLC (for Ibu and Pre).

1.4 Water solubility test of complexes between drugs and Pep fractions A–L

Complexes between drugs and Pep fractions (A–L) were prepared in a similar manner to that described in Section 1.3. An ethanol solution of drugs was prepared by dissolving Indo, Ibu and Pre in ethanol at a concentration of 5.0 g/L. An aqueous solution of Pep was prepared by dissolving 5.0 g/L of Pep fractions (A–L) containing different peptides in water. Equal volumes (500 µL) of the aqueous and ethanol solutions were mixed in a stoppered polypropylene centrifuge tube and shaken using a thermoshaker (MSC-100, Hangzhou Allsheng Instruments) at 30°C for 1 h. After removing the ethanol *in vacuo* using a centrifugal evaporator (Savant SpeedVac SPD1010, Thermo Scientific Inc.), residues were lyophilized to obtain the complexes between the drugs and Pep fractions (A–L).

Table 3 shows conditions for the solubility test of complexes between the drugs and Pep fractions (A–L). The water solubility tests were conducted in a similar manner as those described in Section 1.3. One milliliter of PBS (100 mM) was added to the complexes and the aqueous mixture was shaken at 1,500 rpm at 30°C for 1 h. After filtration of the aqueous mixture using a membrane filter, the concentration of the complexes in the filtrate was determined using reversed phase HPLC.

1.5 X-ray diffraction of complexes between drugs and Pep

The complexes between the three drugs and Pep were prepared in a similar manner to that described in Section 1.3. Indo-Pep and a blank sample (Indo-Brank) were prepared by mixing a 10.7 g/L ethanol solution (5.0 mL) with an aqueous solution (5.0 mL) containing 30.0 g/L of Pep or a blank solution without Pep. A reference complex Indo-Pep-R was prepared by mixing solid Indo and Pep (3 : 7, w/w). Ibu-Pep and a blank sample (Ibu-Blank) were also prepared by mixing a 10.0 g/L ethanol solution (2.5 mL) of Ibu with an aqueous solution (2.5 mL) containing 20.0 g/L of Pep or blank solution without Pep. A reference complex, Ibu-Pep-R, was prepared by mixing solid Ibu and Pep (1 : 1, w/w). Pre-Pep and a blank sample (Pre-Blank) were also prepared by mixing a 1.0 g/L ethanol solution (2.5 mL) of Pre with an aqueous solution (2.5 mL) containing 20.0 g/L of Pep or a blank solution without Pep. A reference complex, Pre-Pep-R, was prepared by mixing solid Pre and Pep (1 : 20, w/w).

The complexes and other reference samples were observed with a scanning electron microscope (VE-8800, Keyence Co.). Their crystal structures were also examined using X-ray diffraction (XRD) with Cu Kα radiation (RINT2000, Rigaku Corp.).

Table 3 Solubility test and analysis conditions of the complexes with Indo, Ibu, and Pre used for different peptide fraction experiments

Solubility test condition	Drug		
	Indo	Ibu	Pre
Vol. of PBS (100 mM) [mL]	1.0	1.0	1.0
pH of PBS (100 mM)	5.5	5.5	5.5
Shaking temperature [°C]	30	30	30
Shaking speed [rpm]	120	120	120
Shaking time [h]	1	1	1
Pore size of membrane filter [µm]	0.20	0.45	0.45
Analytical method	HPLC	HPLC	HPLC
Wavelength of UV-Vis detector [nm]	320	230	245
HPLC condition			
Column temperature [°C]	40	40	40
Mobile phase ratio of ACN/PBS (10 mM)	45/55	45/55	30/70
pH of mobile phase	2.5	2.5	2.5

1.6 Material distribution and particle size analysis

Indo-Pep (250 mg), Ibu-Pep (315 mg) and Pre-Pep (315 mg), prepared as described in Section 1.4, were dissolved in 50, 30 and 30 mL of distilled water, respectively. The aqueous solutions were centrifuged at 3,000×g for 5 min, and supernatants were analyzed (centrifuged samples, F1). Supernatants were filtered through a 0.80 µm membrane filter to obtain filtrates (F2). The filtrates were successively filtered using a 0.45 µm membrane filter and a 0.20 µm membrane filter to obtain fractions F3 and F4, respectively. Furthermore, the fractions F4 were ultrafiltered using UF membranes with MWCOs of 200,000, 50,000, or 10,000 $gmol^{-1}$ to obtain fractions F5, F6, and F7, respectively (Inada *et al.*, 2013; Matsushita *et al.*, 2013; Oshima *et al.*, 2013).

The concentrations of Indo, Ibu and Pre in fractions F1–F7 were determined as described in Section 1.3. The particle sizes of the complexes in fractions F1–F7 were measured using a dynamic light scattering (DLS) particle size/zeta potential analyzer (SZ-100 Nanopartica, Horiba Ltd.).

2. Results and Discussion

2.1 Dependency of water solubility on pH for the three drug complexes with Pep

As shown in our previous study (Inada *et al.*, 2013; Oshima *et al.*, 2013), the water solubility of Indo-Pep increases with increasing pH. The effect of pH on the water solubilities of Ibu-Pep and Pre-Pep was studied to compare ionic and nonionic drugs (**Figure 3**). Figure 3(a) shows the effect of pH on the water solubilities of Ibu-Pep, Ibu-Blank, and Ibu-Pep-R. The water solubility of Ibu-Pep was higher than those of Ibu-Blank and Ibu-Pep-R, while the solubilities of the latter two samples were closely similar. This result suggests that preparation of the complex in solution state is important to obtain a water-soluble complex. The dependence of water solubility on pH for Ibu-Pep was similar to that of Indo-Pep: The solubility increased with increasing

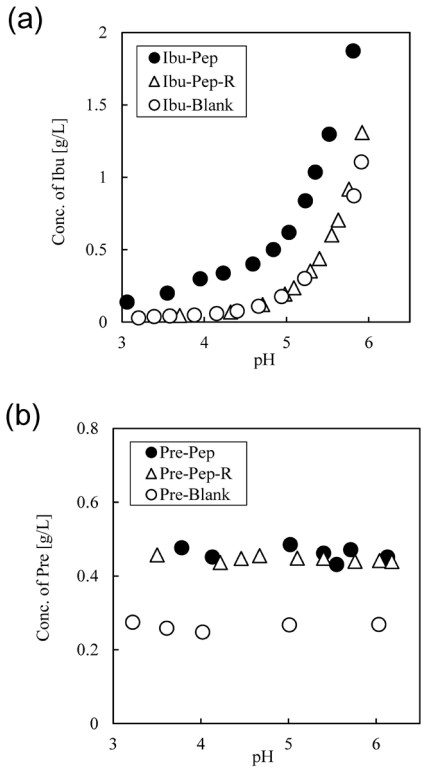

Fig. 3 Dependence on pH of apparent solubilities for (a) Ibu (Ibu, Ibu-Pep-R and Ibu-Pep), and for (b) Pre (Pre, Pre-Pep-R and Pre-Pep)

pH. Indo and Ibu both bear a carboxyl group with pK_a values of 4.5 for Indo (Mahmud *et al.*, 1996) and 4.4–4.5 for Ibu (Hadgraft *et al.*, 2000; Hadgraft and Valenta, 2000). Therefore, deprotonation of the carboxyl groups should influence the solubility of each complex. Recently, ElShaer and coworkers reported that cationic amino acids such as arginine and lysine form ion-pairs with Indo, resulting in enhanced Indo water solubility (ElShaer *et al.*, 2014). Because Pep contains arginine and lysine residues, the dissolution mechanism for Indo-Pep and Ibu-Pep would be similar to that of cationic amino acids.

Figure 3(b) shows the effect of pH on the water solubilities of Pre-Pep, Pre-Blank, and Pre-Pep-R. The water solubilities of Pre-Pep and Pre-Pep-R were higher than that of Pre-Blank, suggesting that complexation with Pep enhances the water solubility of Pre. As the solubilities of Pre-Pep and Pre-Pep-R were similar, the methods used to prepare the complex between Pre and Pep had little or no influence on its solubility. The result differed from the behavior of Indo-Pep and Ibu-Pep. It should be noted that the solubility of Pre-Pep was almost independent of pH, which differs from the behavior of Indo-Pep and Ibu-Pep. The apparent water solubility of the complex between Pep and Ptx is also independent of pH (Inada *et al.*, 2015). Because neither Pre nor Ptx contain dissociable groups, the water solubility of complexes between Pep and most nonionic drugs is independent of pH. However, the apparent water solubility of a complex between albumin hydrolysate and a nonionic compound, coenzyme Q_{10}, depends on the pH (Inada *et al.*, 2013; Matsushita *et al.*, 2013; Oshima *et al.*, 2013), in contrast to the solubilities of Pre and Ptx.

These results suggest that incorporated drugs dominate the dependence of solubility on pH in complexes with Pep. For ionic drugs Indo and Ibu, complexation with Pep enhances water solubility but dissociation of the carboxyl group of the drugs is also important. In contrast, the water solubilities of complexes between Pep and nonionic drugs (Pre and Ptx) are independent of pH. Because peptides are zwitterionic species, the interaction between Pep and drugs would depend upon pH, whereas the water solubility of complexes containing several drugs was independent of pH, so hydrophobic interaction, not electrostatic interaction, would be a dominant factor in the complexation.

Table 4 Concentration of Indo (pH: 5.75–5.97), Ibu (pH: 5.22–5.62), and Pre (pH: 5.71–6.08) in complexes with various polymers

	Indo	Ibu	Pre
Blank	0.098 g/L	0.301 g/L	0.269 g/L
Pep	0.871 g/L	1.298 g/L	0.471 g/L
Glucose	0.148 g/L	0.360 g/L	0.253 g/L
Sucrose	0.156 g/L	0.363 g/L	0.277 g/L
PEG 4,000	0.167 g/L	0.385 g/L	0.475 g/L
PEG 500,000	0.164 g/L	0.375 g/L	0.291 g/L
Arginine	0.134 g/L	1.270 g/L	0.289 g/L
Lysine	0.625 g/L	2.334 g/L	0.264 g/L

2.2 Water solubility of complexes between drugs and various excipients

Table 4 shows the solubility of complexes between three drugs and several different excipients. Saccharides such as glucose and sucrose are effective additives for forming amorphous drugs (Yu, 2001). Polyethylene glycol (PEG) is also used to improve the solubility of poorly water-soluble drugs (Yamashita *et al.*, 2003; Ahuja *et al.*, 2007). Glucose, sucrose, PEG 4,000, and PEG 500,000 were less effective in enhancing the solubility of the three drugs, except for the complex between Pre and PEG 4,000. A solid dispersion including PEG is effective for enhancing the solubility of Pre, as reported by Zakeri-Milani *et al.* (2011). As previously reported (ElShaer *et al.*, 2014), the cationic amino acids arginine and lysine enhance the water solubility of the anionic drugs Indo and Ibu through the formation of ion-pairs. The solubility of the complex between lysine and Ibu is higher than that of the complex Pep and Ibu. In contrast, the solubility of a complex between Indo and Pep was higher than that of a complex between lysine and Ibu. Furthermore, Pep enhances the solubility of Pre, even though arginine and lysine are less effective.

The results show that Pep is effective as an excipient for many kinds of poorly water-soluble drugs. Pep contains both hydrophobic and ionic residues, which contribute to interactions with drugs via hydrophobic and electrostatic interactions. The versatile interactions of Pep with drugs should promote the production of water-soluble complexes,

compared with other simple excipients.

2.3 Water solubility of complexes between drugs and Pep-derived fractions

Pep was fractionated by differences in both hydrophobicity and molecular weight and the three drugs were then complexed with each peptide fraction. The sequences of part of the peptides in each fraction were identified (Inada *et al.*, 2015). **Figures 4(a), (b)** and **(c)** show the solubility of the complexes between the three drugs and Pep fractions (A–L). The solubilities of the complexes of three drugs with all twelve Pep fractions (A–L) were higher than those of the drugs alone. The solubilities of the complexes between Indo and Pep fractions (A–L) depended partially on the hydrophobicity of the Pep fractions. Fractions B, D, E and F, which contained relatively hydrophobic peptides, derived by precipitation using 19 and 27 wt% $(NH_4)_2SO_4$, were more effective in enhancing Indo solubility. However, the solubility of the Indo complex prepared using fraction A was not as high, despite it containing relatively hydrophobic peptides with high molecular weights. This result differs from that for the Ptx complexes with Pep fractions, in which fraction A was the most effective in enhancing Ptx water dispersibility (Inada *et al.*, 2015). For Ibu and Pre, water solubility increased with increasing hydrophobicity of the Pep fractions. However, the solubility also increased when using fractions containing relatively hydrophilic peptides with low molecular weights, such as fraction L. This result suggests that only a specific peptide in Pep does not interact with drugs, but that various peptides would interact with Ibu and Pre. The main driving forces to form the complexes between the three drugs and Pep should be electrostatic and/or hydrophobic interactions, but the complexation mechanism is considered to be quite different for each drug, and peptides that contribute to enhancing the solubility of drugs also differ.

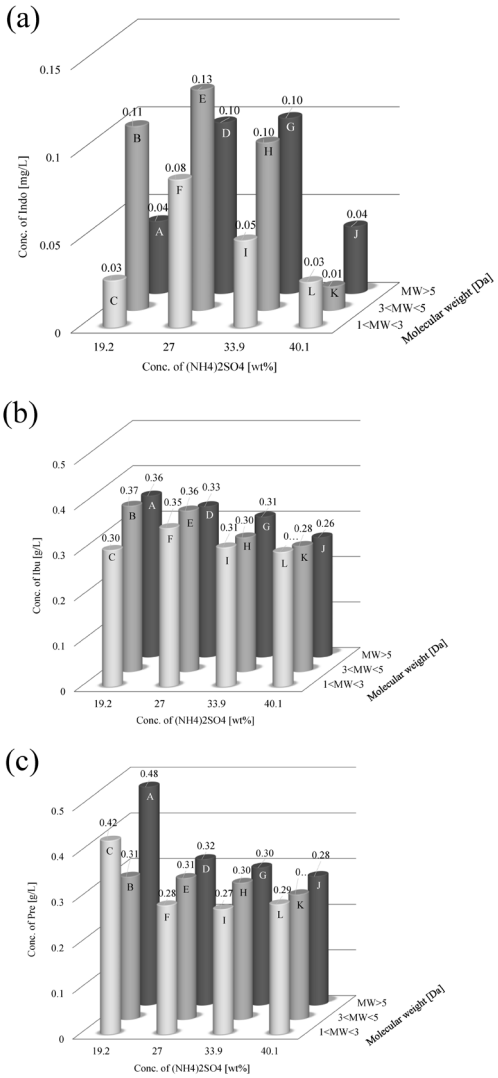

Fig. 4 Apparent solubilities of (a) Indo-Pep, (b) Ibu-Pep, and, (c) Pre-Pep prepared using different Pep fractions

2.4 Characterization of the complexes between drugs and Pep

The XRD patterns for Ibu (Ibu-Blank, Ibu-Pep-R, Pep, and Ibu-Pep) and Pre (Pre-Blank, Pre-Pep-R, Pep, and Pre-Pep) are shown in **Figures 5(a)** and **(b)**, respectively. The XRD pattern of Indo-Blank and Indo-Pep-R showed the presence of a crystalline phase (Inada *et al.*, 2013), while Ibu-Blank and Pre-Blank also showed crystalline peaks. The spectra for Indo-Pep-R, Ibu-Pep-R, and Pre-Pep-R were superimposed on that of Indo-Blank, Ibu-Blank, and Pre-Blank, respectively, and the result agreed with the findings

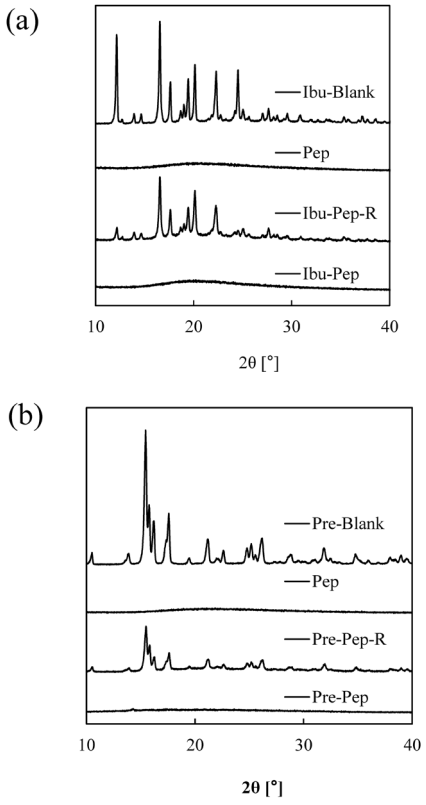

Fig. 5 X-ray diffraction patterns for (a) Ibu (Ibu, Pep, Ibu-Pep-R, and Ibu-Pep), and for (b) Pre (Pre, Pep, Pre-Pep-R, and Pre-Pep)

Table 5 Concentrations of the fractions and relative concentrations for F1 with Indo, Ibu, and Pre

Entry	Indo*		Ibu		Pre	
	Concentration of Indo [g/L]	Conc./Conc.$_{F1}$	Concentration of Ibu [g/L]	Conc./Conc.$_{F1}$	Concentration of Pre [g/L]	Conc./Conc.$_{F1}$
F1	0.415	1	0.448	1	0.250	1
F2	0.423	1.017	0.395	0.880	0.253	1.015
F3	0.410	0.987	0.400	0.891	0.249	0.997
F4	0.405	0.975	0.390	0.870	0.251	1.005
F5	0.409	0.985	0.385	0.858	0.241	0.967
F6	0.377	0.908	0.370	0.825	0.240	0.960
F7	0.346	0.832	0.336	0.750	0.210	0.844

* Inada *et al.* (2013)

for XRD analysis of mixtures of Ibu and Pre kneaded with casein hydrolysate (Kimura *et al.*, 1991; Imai *et al.*, 1998). In contrast, the diffraction peaks of the three drugs were not observed in XRD patterns for Indo-Pep, Ibu-Pep, and Pre-Pep. The results of XRD analysis confirm that the three drugs in the complexes were incorporated in the Pep matrix and were amorphous, supporting the result in Section 2.1. Because the crystalline peaks remained for Indo-Pep-R, Ibu-Pep-R, and Pre-Pep-R, drugs and Pep should be mixed in the solution state.

2.5 Material distribution and particle size analysis

Aqueous solutions of the complexes between the three drugs and Pep were fractionated by centrifugation, filtered using membrane filters (pore sizes ϕ 0.80, 0.45, and 0.20 μm), and ultrafiltered with MWCOs of 200,000, 50,000, and 10,000 $gmol^{-1}$ (Inada *et al.*, 2013; Matsushita *et al.*, 2013; Oshima *et al.*, 2013). **Table 5** summarizes the concentrations of drugs in fractions F1–F7 of the complexes. The Conc./Conc.$_{F1}$ values are the concentration ratio of the drugs in each fraction to that in F1. The concentrations of Indo, Ibu, and Pre in fractions F2–F4 after filtration using the 0.80, 0.45, and 0.20 μm membrane filters was 0.43–0.40, 0.39–0.40, and 0.24–0.26 g/L, which correspond to 97–100%, 87–89%, and 99–100% of the initial solution (F1) concentrations. The relative concentrations of Indo, Ibu, and Pre in fractions F5–F7 after ultrafiltration using the UF membranes with MWCOs of 200,000, 50,000, or 10,000 $gmol^{-1}$ were 83–99%, 75–86%, and 84–97%, respectively. In addition, the particle size of the complexes in fractions F1–F7 could not be measured using DLS, suggesting that the complexes in aqueous media were sufficiently small to pass through ultrafiltration membranes. These results differ from those for complexes between Ptx and Pep; the Ptx-Pep complex is a hydrocolloidal material in aqueous media and the particle size is around 150–200 nm (Inada *et al.*, 2015). Moreover, the coenzyme Q_{10}-albumin peptide complex is also a hydrocolloidal material in aqueous media and the particle size is around 170–280 nm (Matsushita *et al.*, 2013). Thus, the complex between drugs and Pep are present in different states in aqueous media, depending on the type of drug; either being completely soluble (Indo, Ibu, and Pre) or existing as a hydrocolloid (Ptx and coenzyme Q_{10}).

Conclusions

In the present study, ionic (Indo and Ibu) and nonionic (Pre) drugs were complexed with Pep, to enhance their water solubility. Because the dependence on pH of water solubility for the complexes differed according to the type of drug, we conclude that the dependence of solubility on pH is dominated by the drug incorporated in the complex. Pep complexes containing Indo, Ibu, or Pre are relatively small in aqueous media, which differs from those containing Ptx or coenzyme Q_{10}, which exist as hydrocolloids. The results of solubility tests using different Pep fractions show that the solubilities of complexes containing Indo, Ibu, Pre were enhanced using any of the fractions derived from Pep. This result also differs from the result found that for Ptx in our previous study. Therefore, the water solubility and the state of the complex between Pep and drugs in aqueous media differ according to the type of drug incorporated. These findings provide useful insights into applications of protein hydrolysate as an excipient for poorly water-soluble drugs, and for understanding interactions between drugs and peptides.

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