

# Structural Identification of Modified Amino Acids on the Interface between EPO and Its Receptor from EPO BRP, Human Recombinant Erythropoietin by LC/MS Analysis

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Protein modifications of recombinant pharmaceuticals have been observed both *in vitro* and *in vivo*. These modifications may result in lower efficacy, as well as bio-availability changes and antigenicity among the protein pharmaceuticals. Therefore, the contents of modification should be monitored for the quality and efficacy of protein pharmaceuticals. The interface of EPO and its receptor was visualized, and potential amino acids interacting on the interface were also listed. Two different types of modifications on the interface were identified in the preparation of rHu-EPO BRP. A UPLC/Q-TOF MS method was used to evaluate the modification at those variants. The modification of the oxidized variant was localized on the Met54 and the deamidated variants were localized on the Asn47 and Asn147. The extent of oxidation at Met54 was 3.0% and those of deamidation at Asn47 and Asn147 were 2.9% and 4.8%, respectively.

## INTRODUCTION

Recombinant human erythropoietin (rHu-EPO) is a 30,400-Dalton, 165-amino-acid glycosylated protein having more than 40% carbohydrate contents by mass (Davis et al., 1987). rHu-EPO has been used for the treatment of the anemia associated with chronic renal failure over a decade (Eschbach et al., 1989; Winearls et al., 1986). The mode of action for the treatment with rHu-EPO is well-understood. The binding of EPO to specific receptors on cell surface triggers intracellular signaling path-

ways and results in the proliferation and differentiation of cells, and finally to an increase in haematocrit (Lodish et al., 1995).

The interaction between EPO and its receptor is well-characterized by structural determinations using nuclear magnetic resonance (NMR) spectroscopy (Cheetham et al., 1998) and X-ray crystallography (Syed et al., 1998). These studies have proposed the potential amino acids involved in the interactions between EPO and its receptor. There are two binding sites for those interactions. Residues involved in the binding site 1 of EPO are Thr44 and Asn147 hydrogen bonded to the Phe93 of the receptor, and Arg10 and Arg14 of EPO interacting with Met150 of the receptor (Syed et al., 1998).

Any chemical or post-translational modification on amino acids of EPO in the binding sites invariably affects interactions between the EPO and its receptor and may result in lower EPO therapeutic efficacy. In the case of EPO, glycosylation has been well-documented as a post-translational modification. The N-glycosylations are important factors for the stability, pharmacokinetics, and biological activity of EPO (Delorme et al., 1992; Toyoda et al., 2000). However, other modifications have not been well-investigated because of the complex isoform profiles of EPO due to glycosylation.

Biopharmaceuticals are highly complex protein molecules and may have some types of modification during upstream and downstream processes. These changes may influence the safety, purity, potency, and strength of products, which must be well-characterized, controlled, and monitored as part of the manufacturing process (Greer et al., 2002; Walsh, 2010). In the case of EPO, the quality of four different products has been investigated and compared using several bioanalytical techniques (Brinks et al., 2011). All products showed differences in terms of content and potency with the differences in isoform profiles resulting from distinct glycosylation (Brinks et al., 2011). It's been suggested that monitoring of product quality is needed using physicochemical and biological analysis (Brinks et al., 2011).

For the focused characterization of modifications in EPO, we investigated modifications on the binding sites between EPO and its receptor which may affect the efficacy of EPO. To get detailed information on the interface of EPO and its receptor,

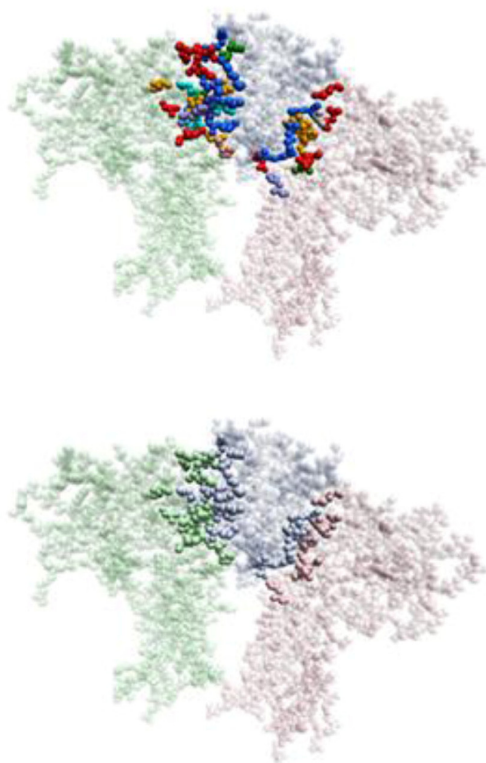
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**Table 1.** Potential amino acids identified on the interface interacting between EPO and its receptors

Binding site	EPO receptor		EPO		Potential modification
	Position	Amino acid	Position	Amino acid	
Site 1	93	PHE	44	THR	Acetylation
	92	SER	45	LYS	
	93	PHE	46	VAL	
	91	SER	47	ASN	Deamidation
	93	PHE	48	PHE	
	33	LEU	48	PHE	Sulfation
	34	GLU	49	TYR	
	34	GLU	52	LYS	
	93	PHE	147	ASN	Deamidation
	204	SER	150	ARG	
	93	PHE	151	GLY	Sulfation
	93	PHE	155	LEU	
	93	PHE	155	LEU	
Site 2	150	MET	10	ARG	Sulfation
	150	MET	11	VAL	
	150	MET	14	ARG	
	93	PHE	15	TYR	Phosphorylation
	91	SER	100	SER	
	62	GLU	103	ARG	
	92	SER	104	SER	



**Fig. 1.** Visualization of the interfaces interacting between EPO and its receptors. PDB ID, 1EER (6) was used for the analysis and potential amino acids on the interfaces were shown high-lighted with different colors representing different amino acids.

we first identified and visualized amino acids involved in the interaction between EPO and its receptor. Afterwards, a UPLC/Q-TOF method was used to evaluate the modifications on the amino acids. This study led to the identification of several modifications on the interface of EPO and its receptor including Asn47, Met54, and Asn147 localized on the binding site 1.

## MATERIALS AND METHODS

### Materials

EPO BRP (EDQM, Ph. Eur.) contains a freeze-dried preparation of erythropoietin in vials with a declared content of 250 µg of erythropoietin per vial. This erythropoietin BRP is known as a 50:50 mixture of epoetin alfa (The R.W. Johnson pharmaceutical Research Institute) and epoetin beta (Boehringer-Mannheim, recently Roche).

Endoproteinase trypsin was purchased from Roche. Sodium phosphate, monobasic and dibasic, DTT, and guanidine-HCl were purchased from Sigma. Acetonitrile (ACN), TFA and methanol were purchased from B&J. Acetic acid was purchased from J.T. Baker.

### Visualization of the interface of EPO and its receptor

To get detailed information on the interface of EPO and its receptor, a visualization tool was developed using ESBTL and OpenGL libraries. Residue information including geometry, chains, and amino acids were extracted from a PDB file loaded with the tool of ESBTL libraries. The distance between amino acids on the interface from different chains was calculated based on the x, y, and z coordinates from the geometry information extracted. The potential amino acids interacting on the interface were visualized based on the cut-off values of the calculated distance information using OpenGL libraries. 1EER PDB file (6) was used to visualize the interface of EPO and its receptor.

**Table 2.** Tryptic peptide identified for rHu-EPO

Peptide	Peptide number	Start	End	Expected mass (Da)	RT (MIN)	Determined mass (Da)	Intensity (counts)	Mass error (ppm)
APPR	T1	1	4	439.254	2.4	439.248	13823	-13.4
LICDSR	T2	5	10	705.348	18.0	705.345	31894	-4.0
VLER	T3	11	14	515.307	5.2	515.302	41869	-8.5
YLLEAK	T4	15	20	735.417	29.1	735.412	78761	-6.0
EAENITGCAEHC SLNENITVPDTK	T5*	21	45	8700.025	41.2	8700.047	2397	2.5
VNFYAWK	T6	46	52	926.465	45.5	926.461	50313	-4.3
R	T7	53	53	174.112				
MEVGQQAVEVW QGLALLSEAVLR	T8	54	76	2525.331	82.8	2525.337	519433	2.5
GQALLVNSSQPW EPLQLHVDK	T9*	77	97	5365.126	52.4	5365.163	51575	6.9
AVSGLR	T10	98	103	601.355	9.9	601.350	45475	-7.2
SLTTLLR	T11	104	110	802.491	41.3	802.489	103380	-3.5
ALGAQK	T12	111	116	586.344	3.6	586.339	43321	-9.2
EAISSPPDAASAA PLR	T13	117	131	2411.970	36.2	2412.008	205918	15.6
EAISSPPDAASAA PLR	T13**	117	131	1464.757	36.2	1464.759	14912	1.2
TITADTFR	T14	132	139	923.471	32.7	923.467	64969	-5.2
K	T15	140	140	146.106				
KLFR	T15-16	140	143	562.359	14.0	562.356	3445	-4.8
LFR	T16	141	143	434.264	10.4	434.260	5578	-8.5
VYSNFLR	T17	144	150	897.471	38.2	897.468	44547	-3.1
GK	T18	151	152	203.127				
LK	T19	153	154	259.190				
LKLYTGEACR	T19-20	153	162	1152.596	36.3	1152.586	1671	-9.1
LYTGEACR	T20	155	162	911.417	25.7	911.413	46068	-4.1
TGD	T21	163	165	291.107				

\*, N-glycopeptide; \*\*, O-glycopeptide

### Peptide mapping with trypsin

The protein sample (100 µg) was reduced and denatured with 1 M DTT and 6 M guanidine-HCl respectively. The denaturation of protein sample was digested with trypsin on a ratio of 1 to 20 (trypsin to protein ratio) for 4 h at 37°C. The obtained peptides were separated using reversed phase chromatography with ACQUITY UPLC system (Waters, UK) equipped with ACQUITY BEH300 C18 column (1.7 µm 2.1 × 150 mm). 10 µl of peptide solutions were injected on the column with 0.2 ml/min flow rate at the temperature of 50°C. Mobile phase A was 0.1% TFA in water and B was 0.1% TFA in ACN with a gradient of 0-50% B in 90 min. The tryptic map was generated by monitoring the absorbance of the effluent with a UV detector at 215 nm. The assignment of each peptide was based on the analysis by an online electrospray ionization mass spectrometry (Q-TOF). MS spectra were obtained with the entire 0.2 ml/min column effluent over a range of 50-3,000 in mass-to-charge ratio with the setting of collision energy at 6 V and cone voltage at 35 V. Calibration of the mass spectrometers with NaCl was performed and [Glu1]-fibrinopeptide B was used in the lockspray channel. The tryptic peptides were identified using BioPharmaLynx Software (Waters, UK) and the sequences of peptides were determined using PLGS Software (Waters, UK).

## RESULTS

### Identification of potential amino acids interacting on the interface of EPO and its receptor

The detailed structural information on the interfaces of EPO and its receptor was visualized using ESBTL and OpenGL libraries. Two binding sites between EPO and its receptor were clearly identified and visualized (Fig. 1). The potential amino acids interacting on the interface were also identified and highlighted in Fig. 1. Amino acid residues localized on AB coil and helix D of EPO were identified on the binding site 1 and residues found on helices A and C of EPO were identified on the binding site 2 (Table 1). Some amino acid residues on the binding site 1 were involved in forming the hydrophobic cap of EPO which was in contact to Phe93 for its receptor by hydrophobic interactions. Two amino acid residues of EPO, Thr44, and Asn147 were known to be hydrogen-bonded with Phe93 of its receptor. Most residues of EPO on the binding site 2 were hydrophilic and forming a hydrophilic pocket flanked by three Arg residues. Potential modifications specific to the amino acid residues are listed on Table 1. Four types of common modification are possible for the residues on the interfaces, including acetylation, deamidation, sulfation, and phosphorylation.

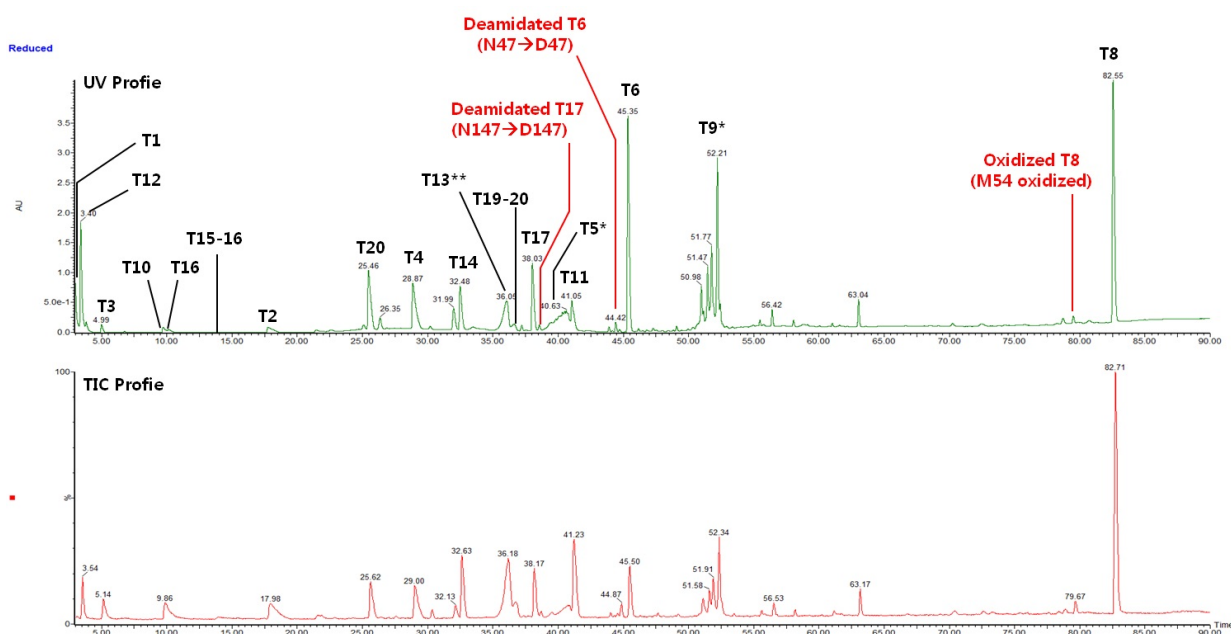


Fig. 2. Tryptic map of rHu-EPO under reduced condition. \*, N-glycopeptide; \*\*, O-glycopeptide.

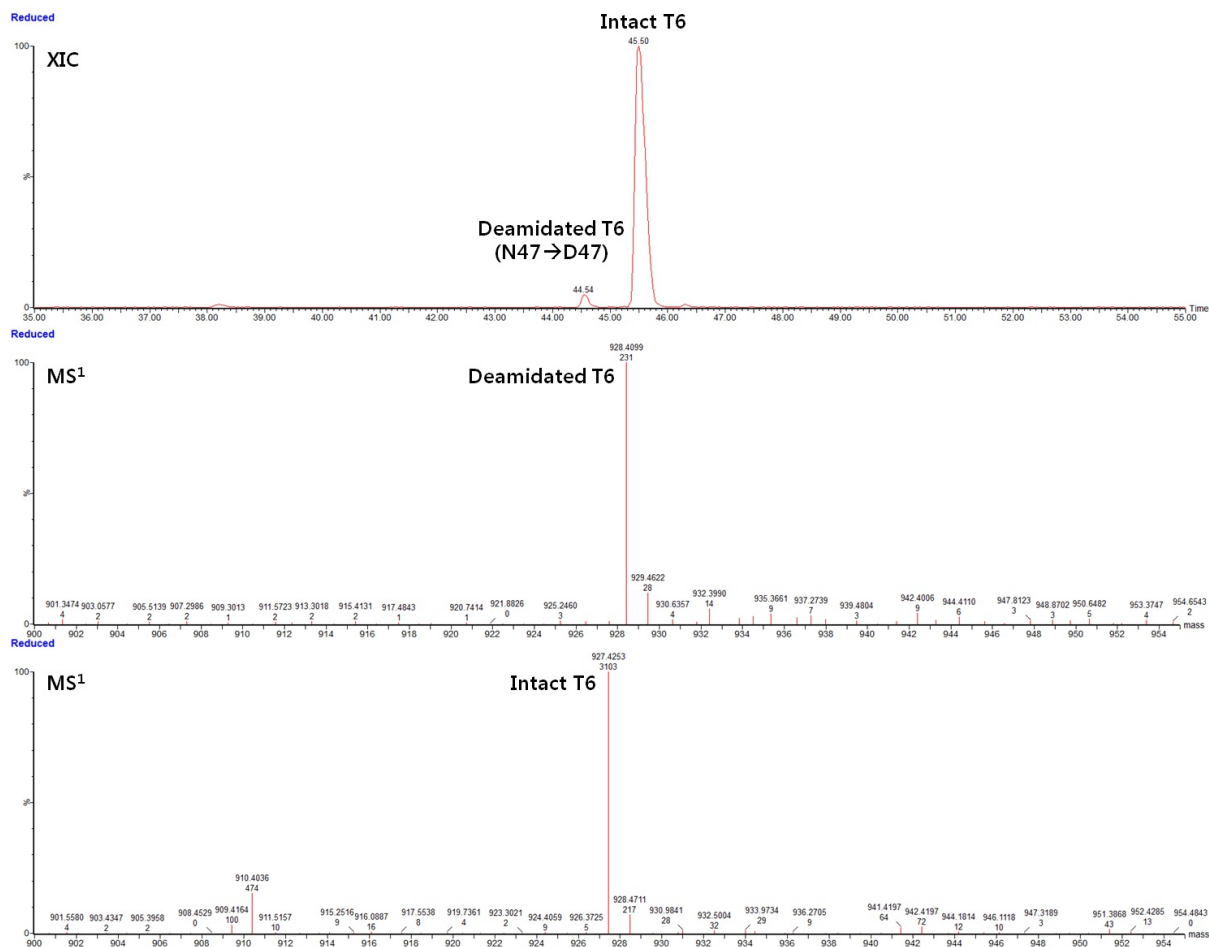
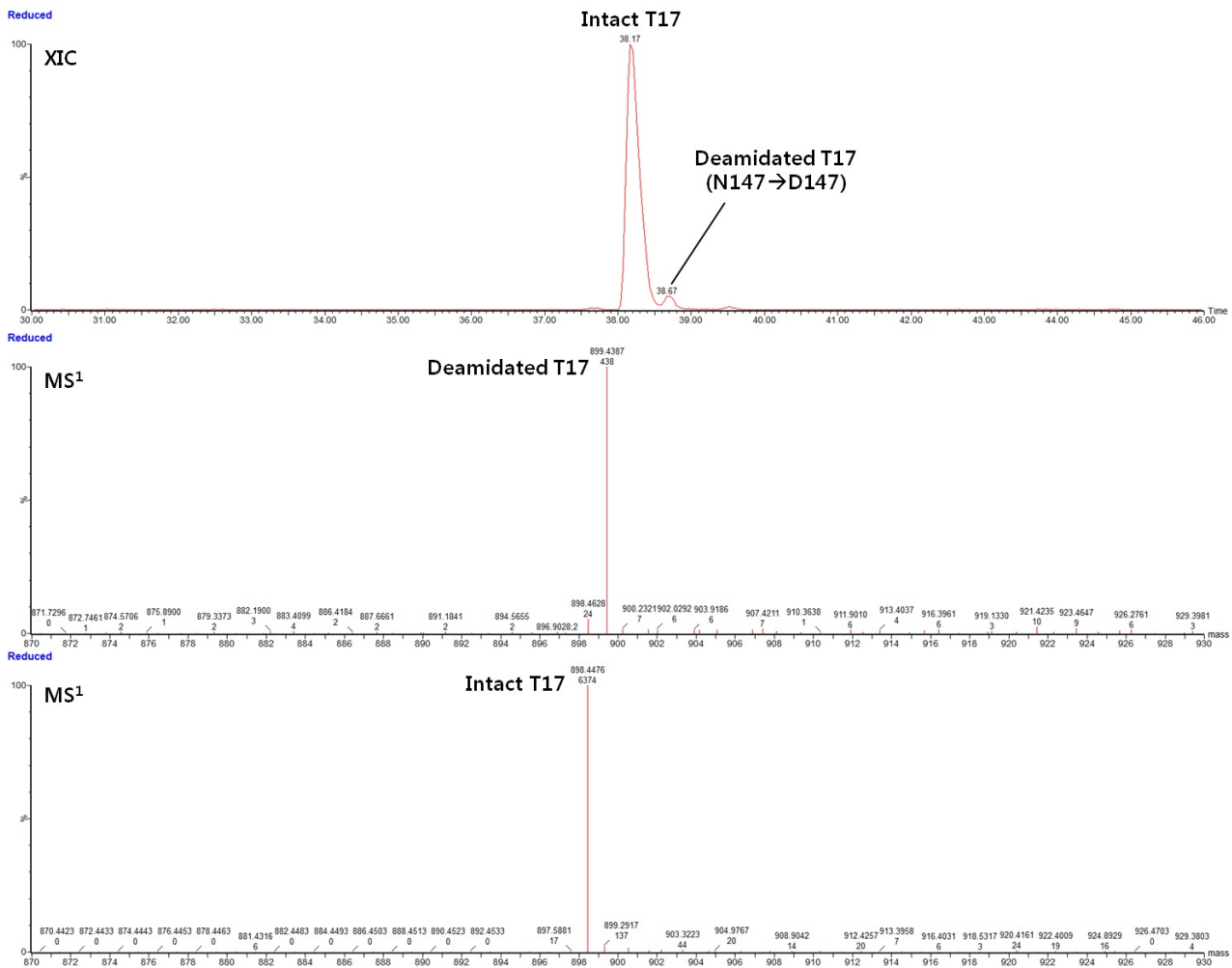


Fig. 3. Identification of deamidation on Asn47 from T6 peptide

**Table 3.** Identification of deamidation at Asn47 of T6 peptide

Peptide	Peptide number	Start	End	Modifiers	Expected mMass (Da)	RT (MIN)	Determined mass (Da)	Intensity (counts)	Mass error (ppm)	% Total
VNFYAWK	T6	46	52		926.465	45.5	926.468	50313	-4.3	97.1
VNFYAWK	T6*	46	52	Deamidation N(1)	927.449	44.6	927.4479	1523	-1.2	2.9



**Fig. 4.** Identification of deamidation on Asn417 from T17 peptide

### Peptide mapping analysis of EPO

In order to get detailed information on any potential modifications, peptide mapping analysis was carried out using endoprotease trypsin and subsequent separation of peptide fragments by RP-UPLC. The tryptic peptides were identified by UV monitoring and online mass spectrometry. The tryptic map was generated based on the assignment of each peptide identified.

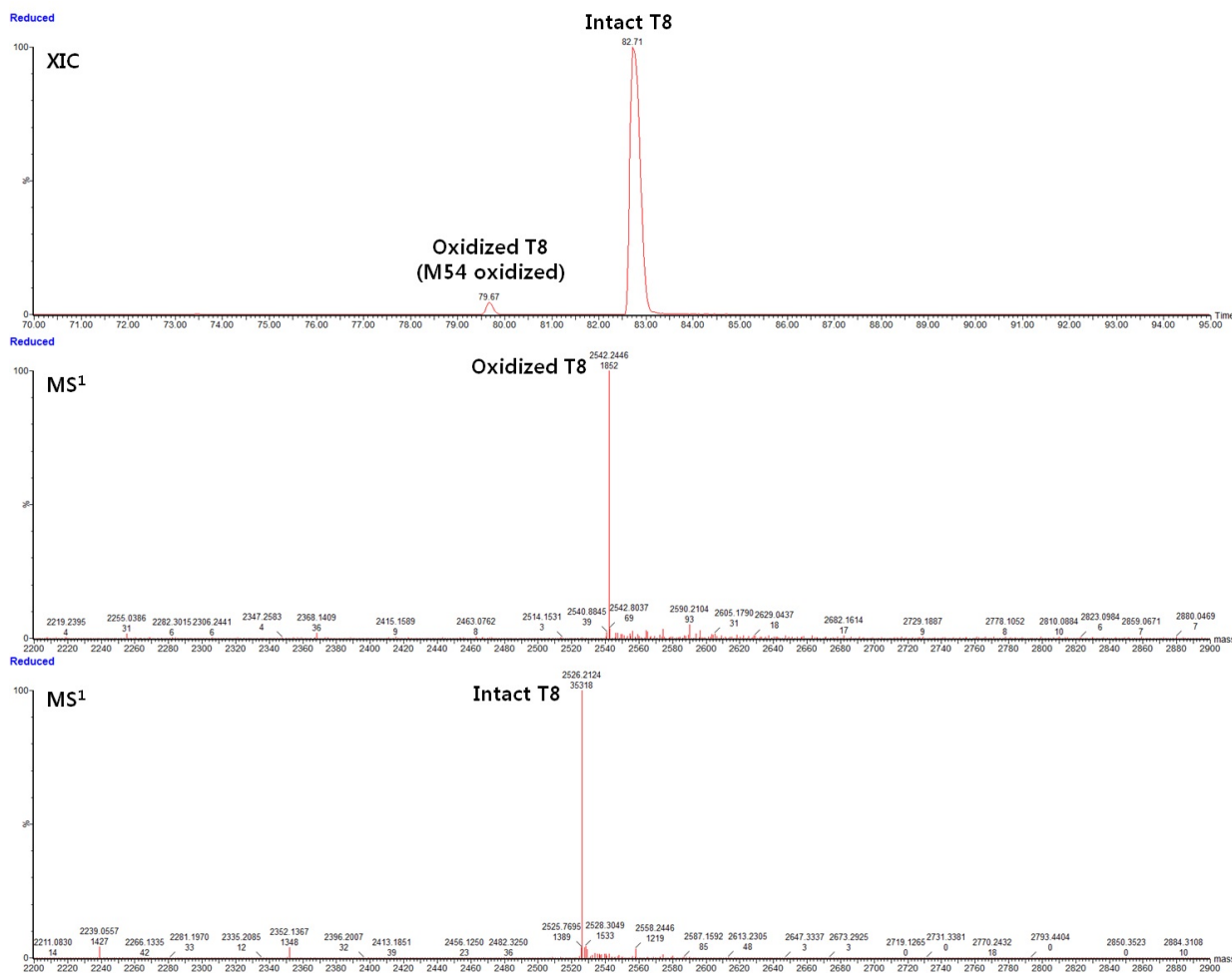
EPO consists of 165 amino acids, and 21 peptides are expected with the digestion of trypsin. T5 peptide with

Asn24/Asn38 and T9 peptide with Asn83 are expected on the glycopeptides with N-glycans. T13 peptide with Ser126 is also expected on the glycopeptides with O-glycans.

A tryptic map of rHu-EPO under reduced condition is shown in Fig. 2 along with TIC profile. The map covers the full sequences of EPO, with the exception of the small polar peptides, T7 ( $^{53}\text{R}^{53}$ ), T18 ( $^{151}\text{GK}^{152}$ ), and T21 ( $^{163}\text{TGD}^{165}$ ). Therefore, the LC-MS of tryptic fragments confirmed 96.4% coverage of the primary sequence for rHu-EPO. Most amino acid residues on the

**Table 4.** Identification of deamidation at Asn147 of T17 peptide

Peptide	Peptid number	Start	End	Modifiers	Expected mass (Da)	RT (MIN)	Determined mass (Da)	Intensity (counts)	Mass error (ppm)	% Total
VYSNFLR	1:T017	144	150		897.4708	38.2	897.468	44547	-3.1	95.2
VYSNFLR	1:T017*	144	150	Deamidation N(1)	898.4548	38.8	898.4493	2257	-6.1	4.8



**Fig. 5.** Identification of oxidation on Met54 from T8 peptide

binding site 1 and 2 were localized on the tryptic map besides Gly151 which was not identified. T6 and T17 peptides having most of the residues from the binding site 1 were identified at the retention time of 45.4 min and 38.0 min on the UV profile of the tryptic map respectively. T3 and T10 peptides having most of residues from the binding site 2 were identified at the retention time of 5.0 min and 9.7 min on the UV profile of the tryptic map respectively (Table 2).

#### Identification of modified amino acids on the interface interacting between EPO and its receptor

The measured masses of the found tryptic peptides were com-

pared to the theoretical masses calculated from the amino acid sequences and the differences were monitored to verify the modifications of amino acid residues on the binding sites. Two deamidated peptides originated from T6 and T17 were detected on the tryptic map during these processes. The deamidated amino acids were further localized on Asn47 and Asn147 which were located on the binding site 1.

The masses of modified T6 peptide were measured and compared to the theoretical masses calculated from the amino acid sequence. T6 peptide had the sequences of  $^{46}\text{VNFYAWK}^{52}$  having one potential deamidation site at Asn47. The expected molecular mass of T6 peptide was 926.47 Da and intact T6



**Table 5.** Identification of oxidation at Met54 of T8 peptide

Peptide	Peptide number	Start	End	Modifiers	Expected mass (Da)	RT (MIN)	Determined mass (Da)	Intensity (counts)	Mass error (ppm)	% Total
MEVGQQ AVEVWQ GLALLSE A VLR	1:T008	54	76		2525.3311	82.8	2525.3334	519433	2.5	97
MEVGQQ AVEVWQ GLALLSE A VLR	1:T008*	54	76	Oxidation M(1)	2541.3259	79.7	2541.3423	15922	6.5	3

peptide having the expected mass was identified at 45.5 min on the TIC profile of the tryptic map (Fig. 3 and Table 3). Deamidated T6 peptide having 927.45 Da which was about 0.98 Da larger than intact T6 peptide was identified at 44.6 min on the TIC profile of the tryptic map. The relative content of deamidated T6 peptide was 2.9% of total (Fig. 3 and Table 3).

The masses of modified T17 peptide were measured and compared to the theoretical masses calculated from the amino acid sequence. T17 peptide had the sequences of <sup>144</sup>VYSNFLR<sup>150</sup> having one potential deamidation site at Asn147. The expected molecular mass of T17 peptide is 897.47 Da and intact T17 peptide having the expected mass was identified at 38.2 min on the TIC profile of the tryptic map (Fig. 4 and Table 4). Deamidated T17 peptide having 898.45 Da which was 0.98 Da larger than intact T17 peptide was identified at 38.8 min on the TIC profile of the tryptic map and the relative content of deamidated T17 peptide was 4.8% of the total (Fig. 4 and Table 4).

Along with deamidation at Asn47 and Asn147, one oxidized peptide originating from T8 was also detected. The oxidized amino acids were further localized on Met54 which was located on the hydrophobic cap of the EPO.

The masses of modified T8 peptide were measured and compared to the theoretical masses calculated from the amino acid sequence. T8 peptide had the sequences of <sup>54</sup>MEVGQQAVEVWQGLALLSEAVLR<sup>76</sup> having one potential oxidation site at Met54. The expected molecular mass of T8 peptide is 2525.33 Da and intact T8 peptide having the expected mass was identified at 82.8 min on the TIC profile of the tryptic map (Fig. 5 and Table 5). Oxidized T8 peptide having 2541.33 Da which was about 16.00 Da larger than intact T8 peptide was identified at 79.7 min on the TIC profile of the tryptic map. The relative content of oxidized T8 peptide was 3.0% of the total (Fig. 5 and Table 5).

## DISCUSSION

Potential amino acid residues were visualized and identified at the interfaces of EPO and its receptor. These were localized on AB coil (residues 44 to 52) and helix D (Asn147, Arg150, Gly151, and Leu155) on the binding site 1 and found on helices A (Arg10, Val11, Arg14, and Tyr15) and C (Ser100, Arg103, and Ser104) on the binding site 2. Mutations on amino acid residues within these two binding sites of EPO prevented interaction to its receptor and finally abolished erythropoietic activities of EPO (Brines et al., 2008; Elliott et al., 1997; Gan et al.,

2012). Thus, any chemical modifications on these residues which were generated during the manufacturing process of rHu-EPO may also affect the efficacy of the final product so the quality issues those modifications should be identified, well-characterized, and monitored.

In order to take a close look at potential modifications at the interfaces, we've first done peptide mapping analysis of rHu-EPO with trypsin and checked if tryptic peptides containing amino acids on the interfaces would be identified. What we found was that most amino acid residues except Gly151 on the binding sites 1 and 2 were localized on the identified tryptic peptides.

Deamidations which converted Asn to Asp were identified at two amino acid residues, Asn47 and Asn147, on the binding site 1. The relative contents of deamidated residues were in the range of 3-5% of the total, which are larger than 1% of total purity and may require detailed characterization for regulation and authorization.

Asn147 of EPO is known to be involved in hydrogen bonding to the Phe93 of its receptor (Syed et al., 1998). Mutational analysis showed that the mutant rHu-EPO on Asn147 to Lys (rHu-EPO[N147K]) reduced affinity for a receptor binding over 100-fold and *in vitro* activity over 200-fold (Elliott et al., 1997). Deamidation on Asn147 seems to be similar effects as the mutant, rHu-EPO(N147K). Deamidation on Asn newly generated negatively charged Asp on that position. This modification may change hydrogen bonding capacity between Asn147 of EPO and Phe83 of its receptor and subsequently affect not only receptor binding activity but also *in vitro* biological activity.

Based on structural data (PDB ID code, 1EER), Asn47 of EPO seemed to be involved in hydrogen bonding to Ser91 of its receptor while the adjacent Lys45 also had the same orientation to Ser91 as Asn47. Mutational data on Asn47 was not available but the mutant rHu-EPO on Lys45 to Asp [rHu-EPO(K45D)] which had the same orientation to Ser91 of its receptor showed 500-fold reduced affinity for receptor binding and 5000-fold reduced *in vitro* activity. The new negatively charged Asp47 by deamidation may produce effects similarly as the mutant, rHu-EPO(K45D).

Along with deamidation, another modification, oxidation, was identified at an amino acid residue, Met54. This modification was identified when rHu-EPO was under stress at pH 9 (Chang et al., 2013). Met54 was placed in the hydrophobic cap of EPO. Along with Met54, several amino acid residues on the binding site 1 were involved in forming the hydrophobic cap of EPO, which consisted of Val46, Phe48, Trp51, Met54, and Leu155 of

EPO. This hydrophobic cap was in contact to the Phe93 of its receptor by hydrophobic interactions. Under the stress, the compact structure of the hydrophobic cap was loosened and Met54 may be vulnerable to be oxidized. Thus, Met54 can be used for a structural indicator of EPO. The content of oxidation on Met54 can be quantified and used for monitoring the structural characteristics of EPO.

The binding of the ligand protein to the specific receptor is major mode of action for most biopharmaceutical therapies. Our studies provide a targeted method focusing on the interface between the ligand and its receptor, which is coupled with UPLC-MS application. This method may contribute a novel and valuable tool which can be used to identify modification hotspots of protein pharmaceuticals.

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