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Preparation of F (ab')$_2$ trastuzumab fragment for Radioimmunoconjugate synthesis of ^{177}Lu-DOTA-F (ab')$_2$-trastuzumab

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Abstract*—Radioimmunodiagnosis or radioimmunotherapy is a technique that has been developed and used in cancer management in recent years.The use of this technique which is based on the use of conjugated-monoclonal antibody (mAb) for imaging or therapy of cancer cells in the last three decades is due to the selectivity and specificity of monoclonal antibodies to the target. One of the antibodies are often used for breast cancer therapy is the anti-HER2 trastuzumab mAb. The use of trastuzumab however has been reported to have a few side effects. One of these effects has been considered to be life threatening, an increasing risk of lung dysfunctional when used was combined with other chemotherapy agent. Based on this fact, an alpha or beta particle emitter/ radionuclide-conjugated trastuzumab has been developed. The radioimmunoconjugate is expected to more be effective to be used either by itself or in its redueced dose. However, the use of radioimmunoconjugate based on a whole mAb for radioimmunotherapy of cancer has been reported to have some weaknesses due to its relatively large size molecule (approximately 150 kD). Thus, it results in a molecule is less effective in reaching the target cells (low pharmacokinetic) and lack of penetration to the target cells. A solution to this problem is by using a radiommonoconjugate derived from enzymatic fragmentation of mAb..This work is a preliminary study to a preparation of ^{177}Lu-DOTA-F(ab')$_2$ trastuzumab. The aim of this work was to prepare trastuzumab F (ab')$_2$ fragment which is going to be used for preparation the above mentioned radioimmunoconjugate. The results of this preliminary work to date were, the optimum time for completely digest of trastuzumab to form F(ab')$_2$ fragment was found to be 18 hrs, where trastuzumab F(ab')$_2$ fragment was able to be purified by using a PD10 column (G25 Sephadex), and purified trastuzumab F(ab')$_2$ fragment which was characterized by SE-HPLC equipped with a Bio-Suite SEC250 column and SDS-PAGE. The result showed a purity of F(ab')$_2$ fragment was carried out of 98% and a molecular weight of approximately 100 kDa respectively.

Keywords*—Trastuzumab, F(ab')$_2$, Fragmentation, Pepsin, Breast Cancer

I. INTRODUCTION

Cancer is a major medical problem and become an important priority in the medical world today. According to the World Health Organization (WHO, 2009), every 11 minutes there is one of world's population died of cancer and every 3 minutes there is a new cancer patient [1]. Indonesian Ministry of Health's report (2010) showed that in 2010, about 6% or 13.2 million population of Indonesia living with cancer and one million deaths were caused by cancer. It was also reported that one of the predominant cancers for women (23%) was breast cancer. Data obtained from Global Cancer Statistics (2002), showed that of the 1,151,298 breast cancer cases caused around 410.712 deaths. Based on these statistics, breast cancer ranks second of the total incidence of cancer occurring worldwide [1].

Radioimmunodiagnosis or radioimmunotherapy was a technique that has been developed and used in cancer management in the last three decades. The use of this technique is based on the use of conjugated-monoclonal antibody (mAb) for imaging or therapy of cancer cells. This technique has been quite attractive to its selectivity and specificity to the target [2], as mAb recognize and bind to antigens or receptors existing on cancer cells. Each mAb is able to recognize a specific antigen on the surface of cancer cells [3,4].

The use of radioimmunoconjugate originated from an intact mAb for cancer management has been reported to have some weaknesses. This is especially due to the relatively large size (approximately 150 kD) of the mAb, thus resulting in a molecule which is less effective in reaching the target cells (*low pharmacokinetics*) and the lack of penetration of the target cell (*poor tumor penetration*) as well as potentially generating an antibody response to the resistance of host cells [6,9,11]. Several studies show that a radioimmunoconjugate originated from an intact mAb such as ^{177}Lu-DOTA-Trastuzumab, took around 24 hrs to properly reach the target [10]. As consequence, a long the way prior the radioimmunonjugate reached its target, unnecessary radiation was exposed to a non-target, healthy tissue. One of the solutions that can overcome this obstacle has been to reduce the size of intact antibodies to smaller forms or fragments, by enzymatic hydrolysis or genetic engineering [11]. Therefore, radioimmunoconjugate that is derived from reduced size or fragmented mAb expected to able reaching its target much faster compared to that of radioimmunoconjugate originated from an intact mAb.

Enzymatic fragmentation of antibody could be easily performed because it does not require complicated steps and the cost is relatively cheap [7]. Several fragment can be prepared from an intact mAb depends on the enzyme use. Fragment bivalent $F(ab')_2$ (Figure 1) can be produced from the breakdown of disulfide bridges on the carbonyl of the cysteine residues by pepsin hydrolysis which will generate Fc fragments and $F(ab')_2$ fragment [8]. Further digestion of mAb with papain will generate monovalent Fab and Fc fragment. The use of papain on an intact mAb meanwhile will directly generate two monovalent Fab and one Fc. In this mechanism papain will breakdown disulfide bridges on $F(ab')_2$ to produce the above-mentioned fragments. The resulting F(ab')2 fragment is composed of two disulfide-connected Fab units. The Fc fragment is extensively degraded, and its small fragments can be separated from F(ab')2 by dialysis, gel filtration or ion exchange chromatography [8].

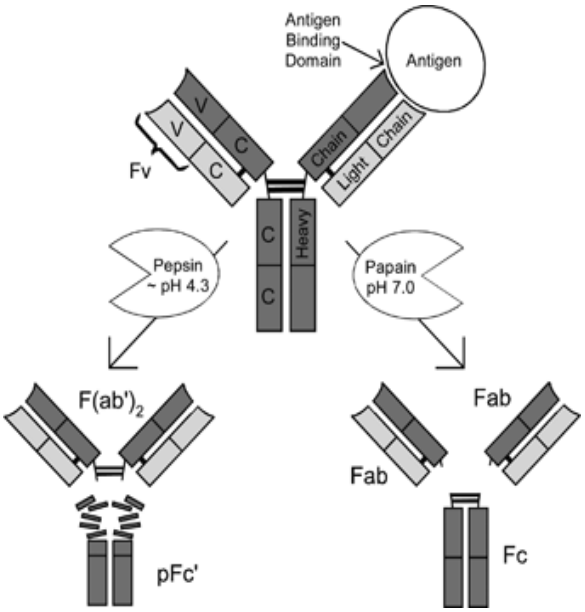


Figure 1. The resulting $F(ab')_2$ fragment is composed of two disulfide-connected Fab units. The Fc fragment is extensively degraded, and it can be separated from F(ab')2 by dialysis, gel filtration or ion exchange chromatography.

The removal of the Fc segment has been reported reducing the non-specific distribution in vivo of the mAb via the Fc receptors found on normal cells. $F(ab')_2$ fragments differ in their pharmacokinetic characteristics compared to the intact antibodies resulting in distinct blood clearance and tumor localization patterns, clearing faster from the circulation than intact antibody while demonstrating better penetration into tumor sites [8]. The rapid clearance from the blood compartment by $F(ab')_2$ results in a higher target-to-non target ratio at earlier time points. A more favorable scenario for the imaging of patients is thus provided. The smaller size and rapid clearance of antibody fragments such as $F(ab')_2$ should also lower their immunogenicity potential, reducing the risk of patients developing a humoral response against the antibody fragment, and potentially permitting repeated treatment of patients [8].

The epidermal growth factor receptor (EGFR) is a target of anticancer therapies because of it's over expression on a variety of malignant epithelial tumors. Several approaches have been used to inhibit the EGFR-associated signal transduction cascade. Monoclonal antibodies that bind to a specific region of EGFR have been

successfully used to inhibit its dimerization and autophosphorylation [13,14]. One of the antibodies that is often used for breast cancer therapy is the anti-HER2 trastuzumab mAb [5]. The use of trastuzumab however has been reported to have a few side effects. One of these effects has been considered to be life threatening, an increasing risk of lung dysfunctional, when its use was combined with other chemotherapy agent [6,8,9]. Based on this fact, alpha or beta particle emitter/ radionuclide-conjugated trastuzumab has been developed. The radioimmunoconjugate is expected to more be effective, therefore it could be used either by itself or in its reduced dose. As consequences, a lower side effect would be expected. However, the use of radioimmunoconjugate originated from an intact mAb for radioimmunodiagnosis or radioimmunotherapy of cancer has been reported to have some weaknesses. The most important one is its lack in of penetrating the target cells. This problem is due a relatively large size molecule (approximately 150 kDa) of an intact mAb. Thus, it results in a molecule which is less effective in reaching the target cells (low pharmacokinetic). A solution that might be offers to this problem is by enzymatic fragmentation of mAb.

The aim of this project is to prepare a bivalent trastuzumab fragment, $F(ab')_2$, which is going to be used as a precursor for preparation a radioimmunoconjugate, ^{177}Lu-1,4,7,10-tetraazacyclotridecane-1,4,7,10-tetraacetic acid (DOTA)-$F(ab')_2$-trastuzumab. By designing the radiolabled ^{177}Lu $F(ab')_2$-trastuzumab, a radionuclide which emits both a beta particle [E_{max} 497 keV (78.6%) and 176 keV (12.2%)] and its gamma emissions [113keV (6.4%) and 208 keV (11%) keV], ^{177}Lu (DOTA)-$F(ab')_2$-trastuzumab is expected to serve as an agent enabling to prevent cancer cell proliferation through energy transfer from the emission of beta particles on cancer cells which ultimately destroys cancer cells. While the emission of gamma radiation used for cancer cell imaging to determine locus, shape and size of the cancer cells [10]. The above-mentioned radioimmunoconjugate, unlike radioimmunoconjugate which is derived from an intact mAb, is expected to demonstrate a better penetration to the target and clearance from circulation which in turn will give an higher target -to-non target ratio at earlier time points.

II. MATERIAL AND METHOD

Chemicals and materials used in this study included commercial Trastuzumab (Herceptin^{TM}) was purchased from Roche, protein standar (Bio-Rad), saline solution (IPHA), sodium acetate, glacial acetic acid, dipotasium hydrogen phosphate, potasium dihydrogen phosphate, sulfo-N hydroxisulfosuccinimide (sulfo-NHS), HCl, NaOH, and EDTA (Merck), bovine serum albumin and sodium azide (Sigma), sodium dodecyl sulfate (SDS) 10%; protein dye (Bio-Rad), gel acrylamide solution (30%T; 2,67C) (Bio-Rad), bis-acrylamide (Sigma), resolving buffer (Tris-HCl 1,5M pH 8,8 (Bio-Rad); stacking buffer Tris-HCl 0,5M pH 6,8 (Bio-Rad); ammonium peroxide disulfate (APS) 10%; N,N,N'N'-tetramethylethylenediamine (TEMED); phosphate-buffered saline (PBS) 0,01 M pH 7,4 and pH 8.0; running buffer (Sigma); staining solution coomasie blue R-250 (Bio-Rad). Another materials used were dialysis cassette 20,000 MWCO (Pierce), protein filter 10,000 MWCO, 5 ml (Viva Science) and Sephadex G-25 column (Pharmacia), Powdered pepsin (Sigma), Tris base, Protein A-Sepharose CL4B (Sigma), polypropilen microcentrifuge tubes (PGC Scientifics).

The equipment used for analysis included high performance liquid chromatography (HPLC) system consists of a pump, a controller, and UV-Vis detector (Shimadzu), 1000 µL sample loop (Rheodine) and Bio-Suite SEC S250 column 7.5 x 300 mm (Waters); single channel scanner (Veenstra Instruments) and Mini Protean gel electrophoresis (Amersham), multi-block heater (Lab Line Inst), Canon digital camera, Fisher Vortex and microcentrifuge (Eppendorf).

Preparation of $F(ab')_2$ tratuzumab was initiated by purification of tratuzumab from its preservatives and excipients by dialysis, which was followed by fragmentation and purification processed. The resulting $F(ab')_2$ tratuzumab was then undergone a molecular weight and purity analysis.

2.1 Purification of Trastuzumab

Trastuzumab was purifed from its preservatives and excipients by dialyzing 20 mg in 0.02M acetate buffer pH 4.5 at 4°C for 72 hours with three buffer changes. The dialysed trastuzumab was then recovered and its concentration was measured by using a UV-Vis spectrophotometer.

2.2 Preparation of $F(ab')_2$ trastuzumab fragment

Pepsin, 2.5mg (3200 U/mg), was added to a solution of 10 mL trastuzumab (5mg/mL) with a ratio of enzyme : antibody 1:4 (mg /ml). Subsequently the mixture was incubated in a Thermomixer with 300 rpm at 37°C for 24 hours. At an interval of 0, 6, 12, 13, 14, 15, 16 and 24 hour, one mL of reaction mixture was taken which was then used for determining the optimum of hydrolysis time. Enzymatic activity was halted with addition 2 mL of 10 mM tris-HCl pH 8.0 to the aliquot. The mixture is stored at 4°C for subsequent purification of $F(ab')_2$ trastuzumab fragment by using a Sephadex G-25 (PD 10) column, with was re-blocked with bovine serum albumin and pre-equilibrated with 0.01 M PBS 7.4.

2.3 Purification of $F(ab')_2$ trastuzumab fragment

Purification of trastuzumab $F(ab')_2$ fragment from other fragments was carried out by using a Sephadex G-25 (PD 10) column (pre-blocked with bovine serum albumin and pre-equilibrated with 0.01 M PBS 7.4).

Aliquot of reaction mixture was loaded onto the top of the column which was then eluted with 0.01 M PBS pH 7.4. Aliquot was eluted in 0.500 mL fractions collectors which were subsequently analysed with an HPLC equipped with Bio-Suite SEC S250 column and by sodium duodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on reducing and non-reducing conditions.

2.4 Analysis of trastuzumab F(ab')$_2$ fragment with HPLC

In order to identify which fraction that contained trastuzumab F(ab')$_2$ fragment, the fractions which were previously retrieved were analysed using an HPLC equipped with size exclusion column, Bio-SEC Suite S250 7.5 x 300 mm (Waters). Flow rate 1 ml / min, with eluent 0.01 M PBS pH 7.4, and isocratic elution mode. A chromatogram from each fraction was then compared to a chromatogram of a gel filtration protein standard.

2.5 Analysis of trastuzumab F(ab')$_2$ fragment with SDS-PAGE

SDS-PAGE was performed by using a Mini-Protean II Slab Cell electrophoresis (Bio Rad). The procedure performed was similar to a standard method reported by Sambrook (1989). A total of 50µL aliquot were mixed with sample buffer (Tris-HCl 1.5M pH 6.8, 6.25% SDS, β-merkaptoetanol, 25% glycerol, 2.5 mM bromophenol blue) with a molar ratio of protein and buffer (2:1). The mixture was then heated for 5 min and centrifuged at 12000 rpm for 3 minutes. Polyacrylamide gels prepared from acrylamide and bis-acrylamide stock solution (30% T, 2.67 C), stacking buffer (0.5 M Tris-HCl pH 6.8), resolving buffer (1.5 M Tris-HCl pH 8.8), 10% SDS, APS and TEMED as the catalyst. After the bottom of the gel (resolving gel) was formed, stacking gel inserted at the top and molds are made for placing the sample protein. Gel formulations for resolving gel was 12% while for the stacking gel was 4%. The SDS-PAGE of samples carried out at a voltage of 150 volts for 60 minutes. As a marker used a protein standard (Biorad Catalog #161-0318) with molecular weight ranged between 7,1 - 209 kDa. Protein staining was performed with Coomasie brilliant blue 0.1% (w / v). The stained gels were then washed with a mixture of methanol: acetic acid solution (40%: 7.5%) and then photographed by Canon digital camera to visualized the protein bands.

III. RESULT & DISCUSSION

This study was a preliminary work for preparation of ^{177}Lu-DOTA-trastuzumab-F(ab')$_2$ fragment. The trastuzumab F(ab')$_2$ fragment which was prepared in this study would be used as precursor for ^{177}Lu-DOTA-trastuzumab-F(ab')$_2$ fragment, a radioimmunoconjugate which is expected to be potential for radioimmunotherapy of cancer. Therefore, the prepared trastuzumab F(ab')$_2$ fragment was expected to have a high purity and properly characterized. Preparation of trastuzumab F(ab')$_2$ fragment was initiated by purification the whole trastuzumab which was commercially available as Herceptin^{TM} by dialysis in order to remove the excipients and other components that might are not needed. The purified trastuzumab was then analyzed with an HPLC which was equipped with SEC Biosuite Column (7.5 x 300 mm) and UV detector.

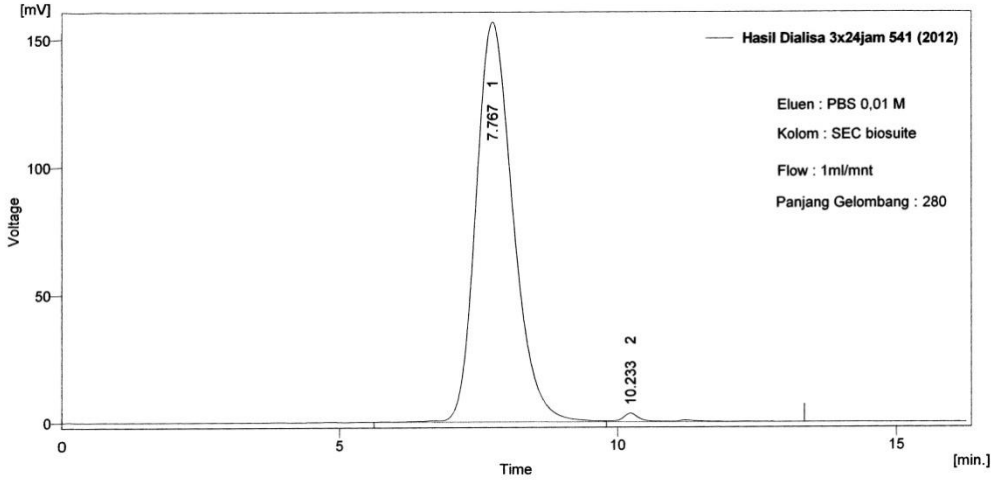


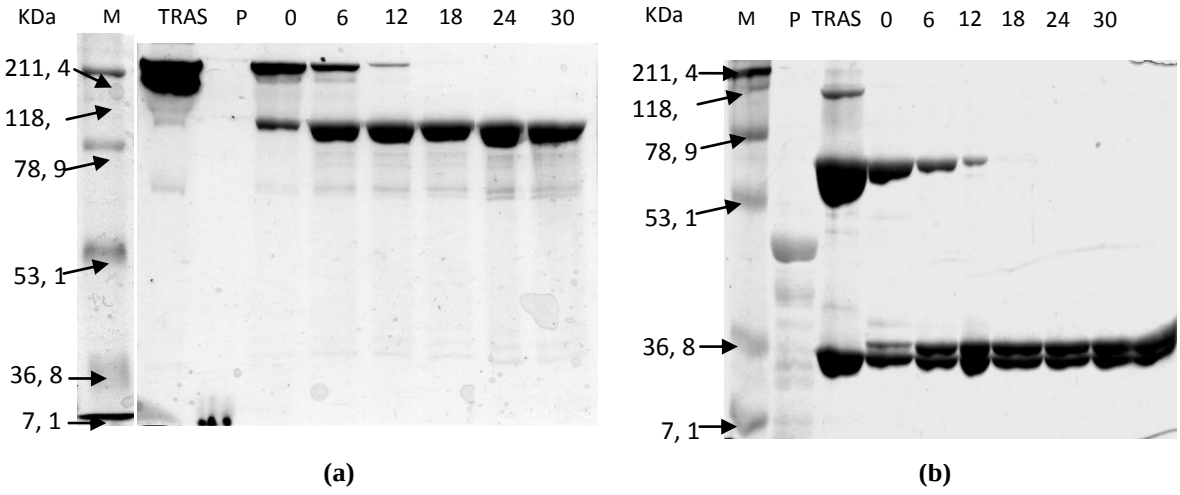
Figure 2. A chromatogram purified of trastuzumab 72 hour

Figure 2 shows the chromatogram of purified trastuzumab. It can be seen from the chromatogram that the purified trastuzumab had a purity of 98,7%. This level of purity was considered to be good enough for further work.

In order to prepare the trastuzumab F(ab')$_2$ fragment, the purified trastuzumab was incubated with pepsin in acetic buffer solution [11]. Pepsin, a nonspecific endopeptidase enzyme that is only active at acid pH. It is irreversibly denatured at neutral or alkaline pH. Digestion of a whole antibody by pepsin normally produces one F(ab')$_2$ fragment and numerous small peptides of the Fc portion as in the Figure 1.

The basic structure of an antibody molecule consists of a "Y"-shaped structure composed of two identical heavy and light chains. Each of these chains contains multiple constant (C) and one variable (V) regions linked by disulfide bonds. The antigen-binding domains reside at the tip of the arms; their effector domains reside in the tail. For most antibodies, these domains can be separated from each other by proteolytic digestion. Under physiological pH, papain is capable of fragmenting all isotypes, irrespective of species, into Fab (monovalent for antigen binding) and Fc (effector domains) fragments by cleaving the heavy chain above the disulfide bonds that hold them together. However, pepsin cuts the molecule below this linkage, giving rise to the $F(ab')_2$ (bivalent for antigen binding) and various fragments of the Fc region, the largest of which is called pFc' (Figure 1)[2].

To determine the optimal (digestion) cleavage time, 2.5mg pepsin (3200 U/mg) was added to a solution of 10 mL trastuzumab (5mg/mL) with a ratio of enzyme : antibody 1:4 (mg /ml) and incubated at 37°C [7]. Aliquots were removed at 0, 6, 12, 18, 24 and 30 hours. As determined by SDS-PAGE, near complete peptic digestion of trastuzumab to a $F(ab')_2$ fragment appears to occur after 18 h (Figure 3). It can be seen in Figure 3 there was a loss of the higher-molecular-weight band of the intact antibody under non-reducing conditions (Figure 3a) and the transition of the heavy-chain band to a lower molecular weight when subjected to reduction with β-mercaptoethanol (Figure 3b).



(a) **(b)**

Figure 3. SDS-PAGE analysis of peptic digest of trastuzumab (a) under non-reducing conditions, (b) reduction with □-mercaptoethanol. (M : Protein standard Biorad, P : Pepsin, TRAS : Trastuzumab).

When aliquot which was 18 hours post digestion analyzed by SDS-PAGE, major bands were visualized corresponding to a molecular weight (MW) of 100 kDa under non-reducing conditions which was associated with the resulting $F(ab')_2$ fragments while two bands were evident at 23 kDa and 22 kDa after reduction (Figure 3b) which were associated with light chains of Fab and Fc fragment. There was no band coreessponding MW of 150 kDa was found in this aliquot which indicated that the whole trastuzumab was completely digested. Lower-molecular weight (LMW) species at approximately 23 and 22 kDa were also evident under non-reducing conditions. These LMW species, with a retention time of 8.51 and 11.46 min on a SE-HPLC, most likely representing light chain of Fab and the Fc fragment as showen in Figure 3b [11]. The retention time of the trastuzumab $F(ab')_2$ was found to 8.51 min when analysed by a SE-HPLC, (Figure 4). This was consistent with a MW of 100 kDa by using protein standard for comparison (data not shown).

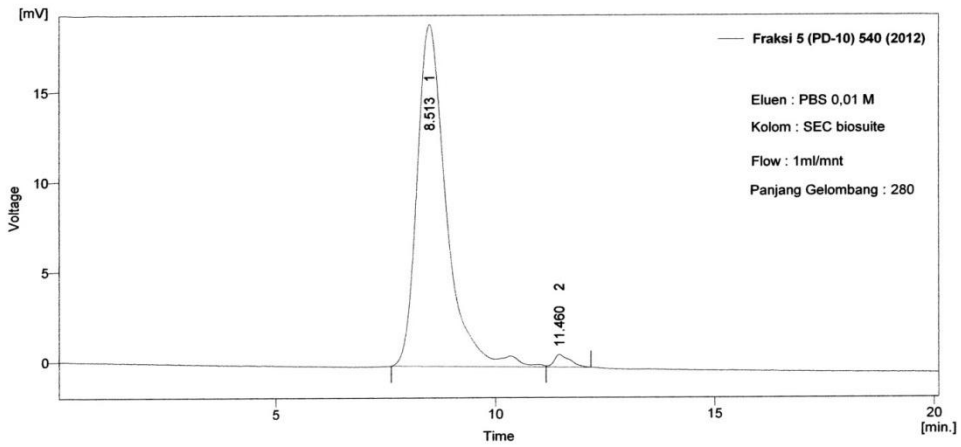


Figure 4. A chromatogram of trastuzumab F(ab')$_2$ fragment after purified using PD10 column

Evaluating the potential of the trastuzumab F(ab')$_2$ for radio immunotherapeutic applications would require larger quantities of the F(ab')$_2$. Therefore, a larger amount of trastuzumab was digested with pepsin with optimum time that was previously established. Prior to its purification pepsin digestic aliquot was also undergone a SE-HPLC. As with the previous preparation, LMW species were also detected by SE-HPLC which comprised approximately 2% of the digest mixture. Purification of trastuzumab F(ab')$_2$ from its LMW species was performed using a PD10 column, made of Sephadex G50, which was pre-blocked with BSA and equilibrated with 0.005 M PBS pH 7.4. Fractions (0.5 mL/ fraction) which was retrieved and monitored with SE-HPLC and SDS-PAGE. Fractions which were associated with trastuzumab F(ab')$_2$ was then pooled and concentrated by using a protein filter Protein A-Sepharose CL4B (Biorad). The final product, which analyzed by SE-HPLC, was found to be comprised of a single product with a retention time of 8.513 consistent with the MW of a F(ab')$_2$ as it can be seen in Figure 4.

IV. CONCLUSSION

The optimum time for fragmentation of trastuzumab by pepsin digestion was found to be 18 hours, in which all the whole antibody has been fragmented into F (ab')$_2$ and Fc. Purification of trastuzumab F(ab)'$_2$ fragment by size exclusion chromatography using a PD 10 column was able to purify the trastuzumab F(ab)'$_2$ trastuzumab from its impurities to give trastuzumab F(ab)'$_2$ fragment with a molecular weight of approximately 100 kDa and purity of 98%.

Based on the exploratory work that has been carried out so far, the following studies are going to performed for the future are to conjugate a bifunctional chelating agent 1,4,7,10-tetraazacyclotridecane-1,4,7,10-tetraacetic acid (DOTA) to tastuzumab F(ab')$_2$ fragments which is then followed by its purification and characterization.. The purified-DOTA)-tastuzumab F(ab')$_2$ fragment is then radiolabel with Lu-177 to produce a radioimmunoconjugate ^{177}Lu-DOTA- trastuzumab F(ab')$_2$ fragment. The purified ^{177}Lu-DOTA- trastuzumab F(ab')$_2$ fragment will then undergo several test *in vitro* as well as *in vivo* such as radiochemical purity, stability and immunoreactivity. Furthermore, to understand the mechanism of interaction of ^{177}Lu-DOTA-F(ab')$_2$-trastuzumab with HER2 a parallel study is also going to be conducted by a silico molecular approach modeling. The results of in silico studies a long with the above-mentioned test will be used as basic information for application of ^{177}Lu-DOTA-F(ab')2-trastuzumab as a radiopharmaceutical candidate for cancer radioimmunotheraphy.

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