# Labeling of the Recombinant Streptokinase Using Iodine-131 as a New Thrombolytic Agent

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### ARTICLE INFO

### ABSTRACT

Stroke and acute myocardial infarction is a disease with the highest mortality in the world. WHO has estimated in 2008, 30% of deaths from heart disease and more than 80% of this cases have been occurred in developing countries. Streptokinase (SK) as an effective thrombolytic agent has been used as a drug of choice for about forty years ago. SK is plasminogen (PG) activator that converts plasminogen to active protease, called plasmin (PN) which degrades fibrin to soluble products. Recombinant streptokinase (SKA) from genetic engineering has been developed at School of Pharmacy ITB to reduce or eliminate immunogenicity of SK. However, scientific disclosures relating to dynamic and its kinetic studies in the body have still to be proven. One method that can explain this phenomenon is the pharmacological studies using radionuclide labeled compounds. Radioiodine labeled compound is used extensively and most suitable for biological studies. This paper describes the preparation of $^{131}$I-SKA and its characterization. The labeling conditions of SKA, such as chloramine-T as an oxidizing agent, amount of SKA, incubation time, and size of resin to purify the labeling yield have been observed. The result showed that the optimum condition of labeling (35.11%) was obtained using 10 μg of chloramine-T and 60 seconds of incubation time. The highest radiochemical purity (97.46 ± 1.14%) has also been obtained by passing through the resin chromatography column using 100 mg Dowex 1x8, size 50-100 mesh. The characterization of $^{131}$I-SKA with SDS PAGE method and autoradiography showed the similar performance with unlabeled-SKA.

## INTRODUCTION

Thrombosis is a condition of the blockage of blood vessel by fibrin clots that cause an acute myocardial infarction and stroke, and becoming leading of death [1]. In 2007, Ministry of Health stated that the highest rate of death has changed from infectious to degenerative diseases, and stroke has the leading causes of death [2]. The incidence of heart diseases has related to lifestyle changes [3].

Thrombolytic agents are the major options than surgery. It works as plasminogen (PG) activator by changing inert PG enzymatically in the fibrinolytic system becomes plasmin (PN) and dissolves the fibrin clot into soluble degradation products, and then degradation products will be phagocyte [1,4]. Several fibrinolytic drugs commonly used are streptokinase (SK), urokinase (UK), and tissue plasminogen activator (tPA) [4,5].

The drug selection is dependent on the price, side effect, severity, in vivo stability, specificity to fibrin, and immunological reactions. UK and tPA are immunological inert, but has a lower in-vivo stability and more expensive than SK that is naturally secreted from *Streptococcus pyogenes* M12 strain CS24 [5].

Biotechnological research at School of Pharmacy ITB has developed several mutants of streptokinase (SKA) to obtain better in vivo activity which is associated with increasing of its biological half life and low immunological reaction. Based on the in-vitro experiments, the observation of pharmacological parameters and in vivo stability has to be explored [6]. Nuclear technique is a method of choice to observe this study. The advantages of this method are more sensitive, simple, and need only small sample to be analyzed. The preparation of SKA labeled iodine-131 as a tracer to be used in this research has been investigated. There are many methods of protein labeling with radioiodine, mostly oxidative reaction. Iodine-131 have been proven to

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be appropriate for labeling both small and large bio-molecules, protein or peptides, well known chemistry, availability easier, and low cost [7].

## EXPERIMENTAL METHODS

The materials used were Iodine-131 as $Na^{131}I$ solution (PT BATAN Technology), Bovine Serum Albumine (TCI), N,N,N',N'-tetramethiletilene diamine (TEMED), coomassie blue R-250 (Pharmacia), protein marker (Amersham), chloramine-T (Sigma), sodium metabisulfite (Merck), Whatman no. 1 paper, and DOWEX 1x8 (Merck). The equipment used were protein electrophoresis (Bio-Rad), paper chromatography apparatus, dose calibrator (RI Deluxe Iisotop Calib II, Victoreen) and single channel analyzer (ORTEC). Streptokinase were obtained from Over-production of *Escherichia coli* BL21 (DE3) consist of pET-32b ska.

## Animals

Male Swiss mice with age of 3 months, and body weight of 40-45 g were used for pharmacokinetic study. Animals used in the experiments received care in compliance with the "Principles of Laboratory Animal Care" and "Guide for the Care and Use of Laboratory Animals" [8,9].

## Over-production of SKA

The SKA Over-production was performed on *E.coli* BL21 (DE3) containing pET-32b. Single colony of *E.coli* inoculated in 10 ml of liquid Luria Bertani broth containing 100μg/ml ampicillin as a selective medium. The culture was incubated for about 18h in a shaking incubator at $37^oC$, and then a total of 10 mL of medium inserted into 200 mL selective medium. Cultures were re-incubated for 3 h at 37°C in a shaking incubator until $OD_{600}$ reached 0.3 to 0.4. Furthermore, the culture was induced with 0.5 mM IPTG and incubation was continued for an additional 3 hours, and then centrifuged at $4^oC$, 4500g for 15 min. Pellet cells obtained from sediment was re-suspended in 5 mL of lysis buffer (50 mM $NaH_2PO_4$ and 300 mM NaCl at pH 8.0) and PMSF to get a final concentration of 1 mM, and lyzed by sonication at the amplitude of 15 for 5 minutes. To prevent temperature elevation, the cells were sequentially sonicated and cooled on ice for several times, each time for 30s. Soluble protein were separated by centrifugation at 12,000 g, 4°C for 15 min, and then purified using nickel affinity column according to the manufacturer's protocol (Protino® Ni-Ted), and characterized by SDS-PAGE. Protein concentration was determined using Bradford method based on coomassie blue staining.

## Labeling optimization of SKA with Iodine-131

SKA were labeled using radioiodine-131. The solution containing 6.2 μg of SKA in 50 μL PBS pH 7.4 was added to the amount of chloramine-T (1 mg / mL) which varied from 10-250μg. The solution was then shaked in a vortex mixer for 30 and 60 seconds at room temperature. To stop the reaction immediately, the sodium metabisulfite solution with a concentration of 2 times the concentration of chloramine-T was added [10,11].

## Purification of $^{131}I$-SKA

Dowex 1x8 (Cl-) was first activated with 1 N HCl solution for 1 hour, then washed with water until neutral pH. Three different weights of Dowex (100, 500 and 1,500mg) was inserted into the column with a ± 1cm diameter, and rinsed with 0.02 M phosphate buffer pH 7.5. The labeled $^{131}I$-SKA was passed to the Dowex column and then eluted with 0.02 M phosphate buffer pH 7.5 containing 1% BSA and 0.1% KI [10]. 0.5-mL of each fraction was collected and measured with a dose calibrator. The radiochemical purity was then determined by using chromatography system.

## Radiochemical purity determination of $^{131}I$-SKA

Radiochemical purity was determined by ascending paper chromatography method using stationary phase Whatman no. 1, and the mobile phase of methanol-water (85:15). Paper chromatogram was dried in oven at 80°C for 5 minutes and then every 1 cm piece of paper is measured by the single-channel gamma counter detector NaI (Tl). Retention factor $(R_f)$ of $^{131}I$-SKA = 0.0 and $R_f$ of iodide-131 = 0.6 to 0.9 [12].

## Characterization of $^{131}I$-SKA

Characterization of $^{131}I$-SKA was determined using Sodium Polyacrylamide Gel Electrophoresis (SDS PAGE). A precisely 8 μL sample of $^{131}I$-SKA and 2 μL loading buffer inserted into the well

containing 10% acrylamide gel. The first gel stained with coomassie blue to determine the band formed while the second gel was exposed to X-ray films for 4 hours in the darkroom. The film was washed with a fixer solution and developer respectively for 15 minutes. Intensity that appears on autoradiography compared with SKA bands.

## Stability of ^{131}I-SKA

After labeling with iodine-131, the solution was kept in a container at room temperature ($25^oC$), $37^oC$, $-4^oC$ and $-20^oC$. Radiochemical purity of ^{131}I-SKA examined with a variety of time by ascending paper chromatography method in accordance with the above procedure.

## RESULTS AND DISCUSSION

The optimum results of SKA Over-production obtained by using 0.5 mM IPTG as an inducer for 3 hours at $37^oC$. The yield of pellet cell was 0.956 g of 200 mL culture (0.478% g/mL). Crude extract obtained at this stage, still contains many impurities from the host protein E. coli and from the medium. Purification of SKA has been conducted using nickel affinity column chromatography. Fusion protein of SKA containing histidine chain initially bound to the nickel column and will be released when it was eluted using imidazole solution [13]. The highest amount of SKA has been found in the $2^{nd}$ eluat. The results then characterized by SDS-PAGE and showed a thick band at 63.8 kDa (Fig 1).
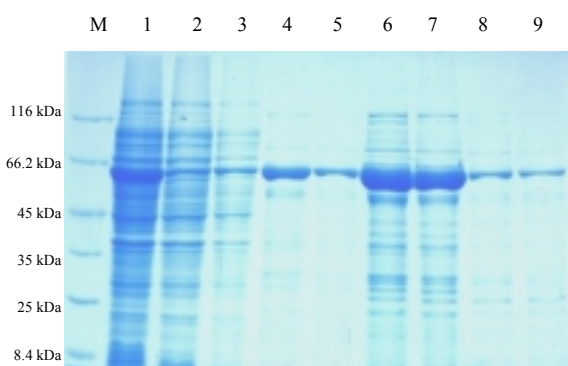


**Fig. 1**. Electroforegram of SKA purified by SDS-PAGE. Protein marker (M); Crude protein extract (1); Flow Through (2); Washing (3); First Eluate (4-5); $2^{nd}$ Eluate (6-7); $3^{rd}$ Eluate (8-9).

Labeled compound with γ-emitter, such as radio-iodinated compounds, have much wider applications considerably in medical, biochemical, and other related field [14]. Among of all iodine

radioisotopes, ^{131}I is most suitable for in vivo studies because it has a convenient half life (8 days) and 364 keV photons energy and gives low radiation dose [15]. In the oxidized form, iodine binds strongly to various molecules. In protein iodination, the phenolic ring of tyrosine is the primary site of iodination. And, in this case, SKA has as many as 22 pieces of tyrosine rings by pyMOL software as shown in Fig. 2.

One of the method commonly used in radioiodination is the "chloramine-T method". Chloramine-T is a sodium salt of N-monochloro-p-toluene-sulfonamide and is a mild oxidizing agent. Chloramine-T oxidizes iodide to a reactive iodine species, which then labels the compound [14]. The reaction occurs through electrophilic substitution on the aromatic tyrosine chain in SKA [16,17]. Iodine is bound to positively charge ortho position of the group monoiodo or diiodotyrosine as shown in Fig. 3 [16,17].
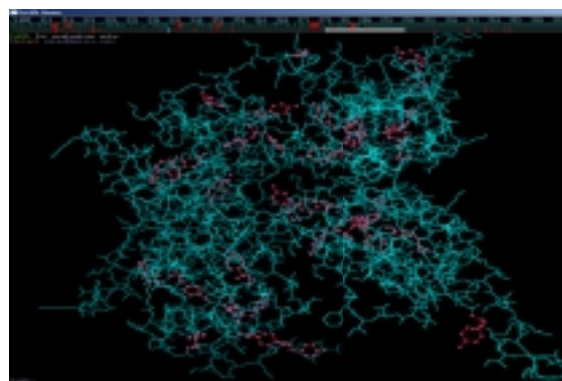


**Fig. 2**. Ring of tyrosine in the crystal structure of Streptokinase-A.



tyrosine          monoiodo tyrosine
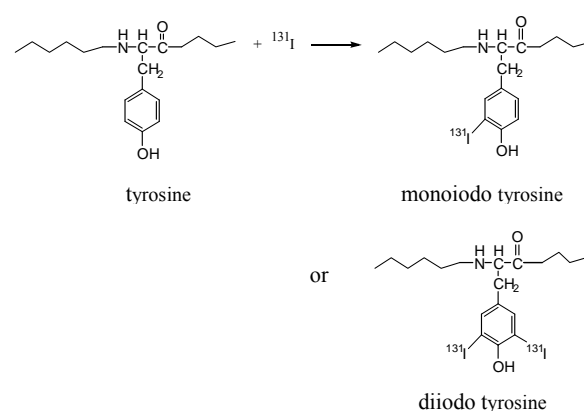
or

diiodo tyrosine

**Fig. 3**. Principle of Protein Radioiodination [17,18].

A number of analytical methods are used to detect the radiochemical impurities in radio-labeled compounds. In this experiment, the purity of ^{131}I-SKA has been determined by ascending paper chromatography system using Whatman paper no.1

as the stationary phase, and a mixture of methanol : water (85 : 15) as the mobile phase [12]. Each component is characterized by an Rf value, which is defined as the ratio of the distance traveled by the component ($^{131}$I-SKA and its impurities) to the distance the solvent advanced from the original point of application of the test material (Fig. 4). Radiochemical purity of $^{131}$I-SKA was calculated based on the count numbers of the peak area of $^{131}$I-SKA (Rf = 0) divided by total counts of the peak area of $^{131}$I-SKA and the peak of impurities (Rf = 0.6 to 0.9) as follow: (Number of radioactive counts of $^{131}$I-SKA / Number of total activity) x 100%
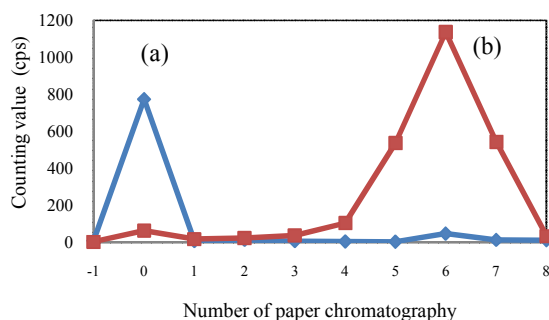


**Fig. 4**. Chromatogram of (a) $^{131}$I-SKA using Whatman no. 1 (stationary phase) and methanol-water 85:15 (mobile phase) and (b) Na$^{131}$I.

A high labeling yield is always desirable, although it may not be attainable in many cases. Several parameters influenced were varied in order to get optimum condition in SKA labeling. The role of chloramine-T is very important in the radioiodination labeling; however, chloramine-T is a highly reactive substance and can cause denaturation of proteins [14]. The excess oxidizing agent can easily oxidise the molecule beeing labeled, and the addition of sodium metabisulfite can stop the reaction immediately. Table 1 showed the amount of SKA and chloramine-T used in the labeling. The highest labeling yield was obtained with molar ratio between chloramine-T : SKA = 1 : 452 and 60s of incubation time.

**Table 1**. Optimization of Chloramine T and incubation time  of $^{131}$I-SKA labeling.

|  | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| SKA (μg) | 6.2 | 6.2 | 6.2 | 6.2 | 6.2 | 6.2 | 6.2 |
| Chloramine T (μg) | 150 | 100 | 50 | 250 | 150 | 50 | 10 |
| Incubation time (s) | 30 | 30 | 30 | 60 | 60 | 60 | 60 |
| Labeling Yield (%) | 7.88 | 8.45 | 9.29 | 7.90 | 9.09 | 10.82 | 35.11 |

Irrespective of the radio-labeling methods, the prepared product will require purification before use. To retain the enhanced sensitivity and precision of the biomedical studies, the purity of the label compound needs to be high. Radiochemical and chemical impurities arise from incomplete labeling of compounds and can be separated by various methods. For this reason, the $^{131}$I-SKA as a labeled product was immediately separated from impurities by ion exchange chromatography method. Dowex 1x8 was selected resin that suitable for this purpose. Ion exchange was performed by passing the labeled product through a column ionic resin (Dowex 1x8) and eluting with phosphate buffer. The resin sizes and the diameters of the column were varied in order to obtain high radiochemical purity as shown in Table 2. The elution can be considerably improved by the presence of sufficient carrier protein (BSA) in the eluting buffer. The result showed that the optimum purification condition (97.7%) obtained by using 100mg Dowex 1x8, 50-100 mesh and 0.5 cm diameter of column.

**Table 2**. Optimization of purification $^{131}$I-SKA using DOWEX 1x8.

| Size colums (mesh) | Resin (mg) | Diameter (cm) | Radiochemical purity (%) | |
|---|---|---|---|---|
|  |  |  | previous | final |
| 100-200 | 500 | 1.5 | 9.29 | - |
| 100-200 | 500 | 0.5 | < 5 | 66.67 |
| 50-100 | 1500 | 1.5 | 18.59 | 93.31 |
| 50-100 | 100 | 0.5 | 18.95 | 97.70 |

The purification profile of $^{131}$I-SKA, as shown in Fig. 5 and Table 3, illustrates the fraction number of elution. Radiochemical purity was decreased at the 3$^{rd}$ until the 5$^{th}$ fraction (<50%). The highest, both of radioactivity and radiochemical purity was found in the 2$^{nd}$ fraction.
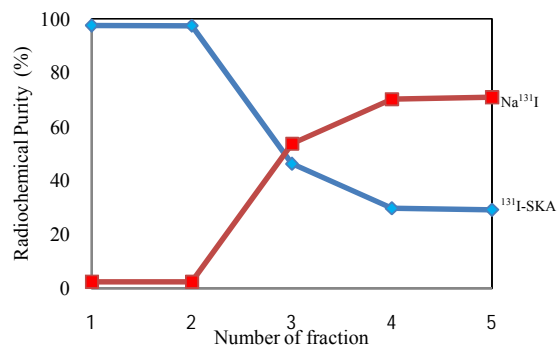


**Fig. 5**. Purification profile of $^{131}$I-SKA using Dowex 1x8, 50-100 mesh, and 0.5cm of column diameter.

**Table 3**. Radioactivity of ^{131}I-SKA fraction.

| Number of fraction | Radioactivity ^{131}I-SKA (mCi) |
| --- | --- |
| 1 | 0,005 |
| 2 | 0,967 |
| 3 | 0,055 |
| 4 | 0,044 |
| 5 | 0,039 |

Sodium Dodecyl Sulphate Polyacrilamide Gel Electrophoresis (SDS PAGE) and autoradiography method has been used to characterize ^{131}I-SKA labeled compound. Based on SDS PAGE analysis indicated that ^{131}I-SKA bands parallel to unlabeled SKA with molecular weight of 63.8 kDa as also shown by autoradiography method in Fig. 6. This result proved that SKA has been labeled with iodine-131, and has not degraded during and after labeling.
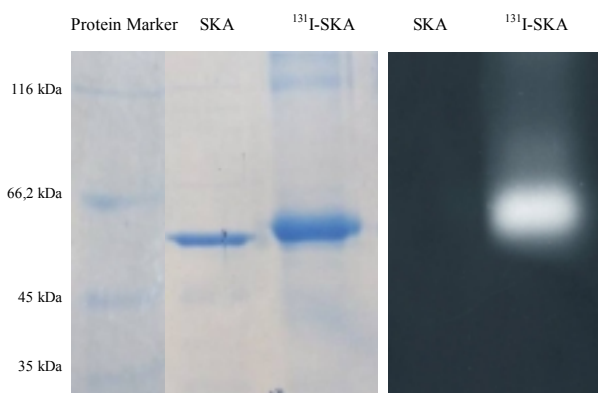


**Fig. 6**. Characterization of ^{131}I-SKA SDS PAGE and autoradiography.

In general, a labeled compound has a self life during which it can be used safely for its intended purpose. The loss of efficacy of a labeled compound over a period of time may result from radiolysis and depends on the physical half life of the radionuclide, the nature of emitted radiations, the solvent, any additive, pH, and the nature of the chemical bond between the radionuclide and the molecule [10,14,15]. In this experiment, the stability of labeled ^{131}I-SKA has been observed in various temperature, at 25°C, 37°C, 4°C and -20°C. As shown on Fig. 7, ^{131}I-SKA was stable at 25°C and 37°C for about 3 hours; however, the stability profile at 37°C was lower than the product stored at room temperature, it may be the increase of temperature will affect the release of iodine atom from tyrosine chain of SKA [19]. The instability process was also shown by the

decreasing of radiochemical purity after 3 days storage at 4°C and -20°C. On the second days, the radiochemical purity of SKA stored at -20°C (95.29%) still indicated better than 4°C (92.24%). ^{131}I-SKA can be stored for about 2 days at -20°C with radiochemical purity more than 95%.
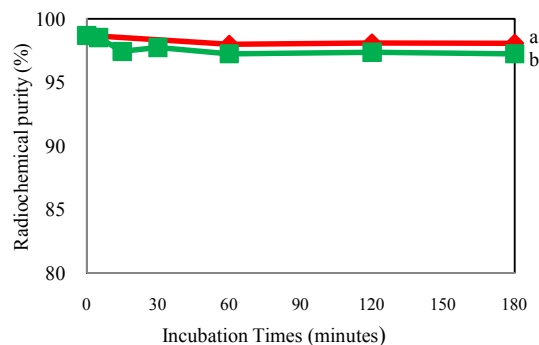


**Fig. 7**. Stability profile of ^{131}I-SKA at (a) 25°C and (b) 37°C.
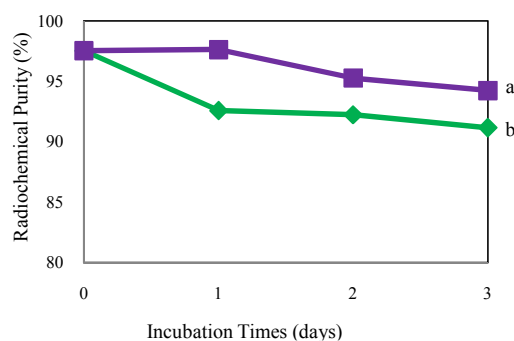


**Fig. 8**. Stability profile of ^{131}I-SKA at (a) -20°C and (b) 4°C.

## CONCLUSION

The recombinant streptokinase (SKA) has been successfully labeled with radioiodine-131 using the chloramine-T method. The labeling yield was about 35% with radiochemical purity more than 95%. The labeling yield was not very high, but the method allows iodination without alteration of SKA. The characteristics of ^{131}I-SKA are suitable and can be used for biological studies such as in pharmacological trials using animal models or in other preclinical assessments.

## ACKNOWLEDGMENT

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