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Effects of β-Mercaptoethanol and Hydrogen Peroxide on Enzymatic Conversion of Human Proinsulin to Insulin

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Human insulin is a hormone well-known to regulate the blood glucose level. Recombinant preproinsulin, a precursor of authentic insulin, is typically produced in *E. coli* as an inactive inclusion body, the solubilization of which needs the addition of reducing agents such as β-mercaptoethanol. To make authentic insulin, recombinant preproinsulin is modified enzymatically by trypsin and carboxypeptidase B. The effects of β-mercaptoethanol on the formation of human insulin derivatives were investigated in the enzymatic modification by using commercially available human proinsulin as a substrate. Addition of 1 mM β-mercaptoethanol induced the formation of various insulin derivatives. Among them, the second major one, impurity 3, was found to be identical to the insulin B chain fragment from Phe_1 to Glu_{21}. Minimization of the formation of insulin derivatives and concomitant improvement of the production yield of human insulin were achieved by the addition of hydrogen peroxide. Hydrogen peroxide bound with β-mercaptoethanol and thereby reduced the negative effects of β-mercaptoethanol considerably. Elimination of the impurity 3 and other derivatives by the addition of over 10 mM hydrogen peroxide in the presence of β-mercaptoethanol led to a 1.3-fold increase in the recovery efficiency of insulin, compared with those for the case without hydrogen peroxide. The positive effects of hydrogen peroxide were also confirmed with recombinant human preproinsulin expressed in recombinant *E. coli* as an inclusion body.

Keywords: Human insulin, β-mercaptoethanol, hydrogen peroxide, insulin derivative, recombinant *E. coli*

Insulin is a hormone secreted in the pancreas to regulate the glucose level in blood. It binds to insulin receptors on the cell surface, thereby promoting glucose usage and reducing the blood glucose level. It has been used as a therapeutic agent of diabetes [9]. Human insulin with 51 amino acids is composed of two polypeptide chains (A and B), which are linked by two disulfide bonds. The A chain contains 21 amino acids and an intramolecular disulfide bond between Cys_6 and Cys_{11}, whereas the B chain has 30 residues (Fig. 1). Initially, insulin is produced in the human body as a nonprocessed form, preproinsulin, which contains 24 amino acids of a signal peptide at the N-terminus of insulin. When preproinsulin passes through membranes, it is converted to a final product, insulin [11]. As the first recombinant DNA protein for therapeutic use, recombinant human insulin has been successfully expressed

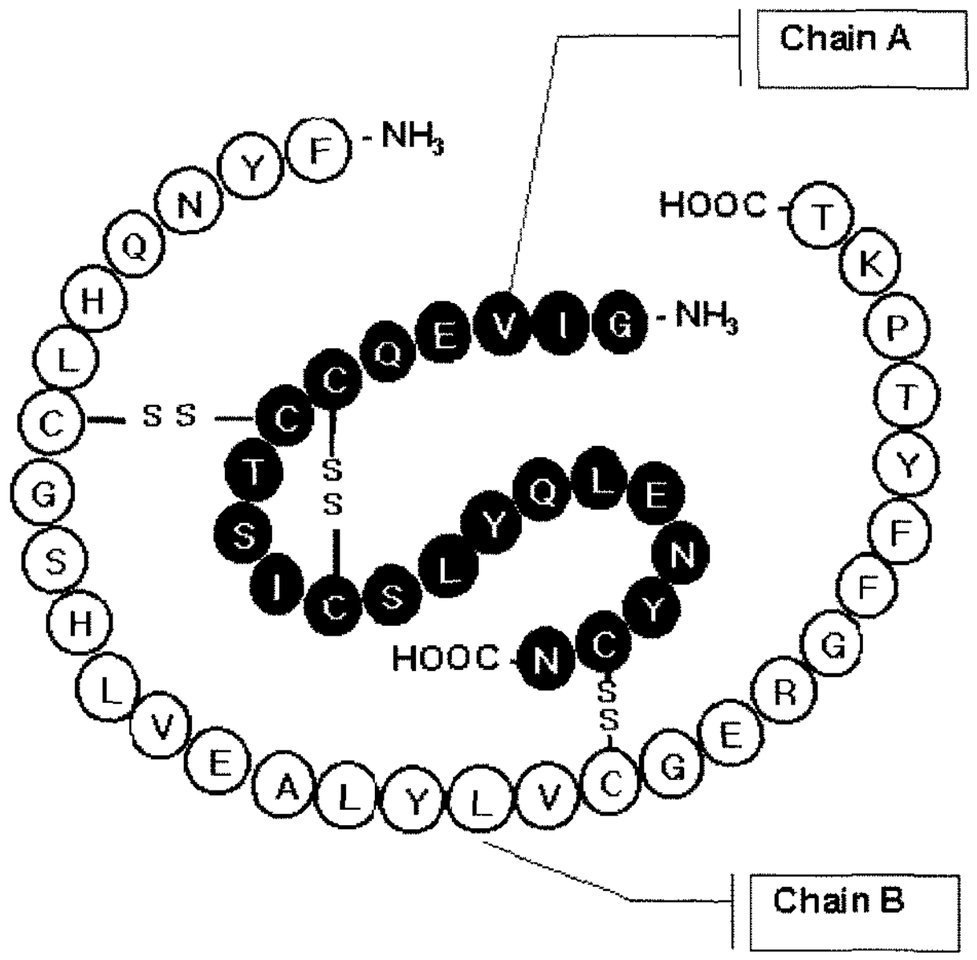

Fig. 1. Structure of human insulin.
The black chain indicates the human insulin A chain and the white one shows the human insulin B chain. The alphabet inside each circle is a one-letter symbol for an amino acid.

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in *E. coli* systems [13, 16]. Unlike the preproinsulin originated from the human body, some types of recombinant preproinsulin containing diverse synthetic peptides at its N- and/or C-terminus were stably expressed in *E. coli* [1, 2].

The overexpression of heterologous proteins in *E. coli* often results in the formation of insoluble aggregates called inclusion bodies [6, 8, 10]. Native or modified insulin was usually produced as an inactive inclusion body in recombinant *E. coli* at a considerable level [1, 16, 19]. For the recovery of the active form from inclusion bodies, several additional steps are required. After cell homogenization and moderate centrifugation, the collected inclusion bodies are solubilized with high concentrations of denaturants such as urea and guanidine hydrochloride [3, 18]. In most cases, reducing agents cleave a disulfide bond between cysteine residues and prevent the formation of non-native intra- and inter-molecular disulfide bonds in highly concentrated protein solutions at alkaline pH. Moreover, they enhance refolding efficiency by reducing any misformed disulfide bonding and air oxidation [3, 14]. β-Mercaptoethanol (MCE), dithiothreitol (DTT), and dithioerythritol (DTE) are typically used as reducing agents. The reduced and unfolded form of a target protein is then refolded in order to recover its native structure and biological activity [20].

The refolded preproinsulin or proinsulin is converted to insulin by *in vitro* enzymatic modification with an enzyme mixture of trypsin and carboxypeptidase B [5]. Trypsin cleaves the peptide bond between lysine and arginine at the C-terminal side of the recombinant proinsulin. Carboxypeptidase B successively cuts off a basic amino acid at the carboxyl terminal, which is newly formed by the trypsin cleavage. The final products of the enzyme reaction are insulin and free C-peptide [4].

Heterologous expression of target proteins by recombinant DNA technology often caused the undesirable production of their derivatives by proteolysis, deamidation, methionine oxidation, and covalent attachment [15]. Most human insulin derivatives are made during the enzymatic modification of proinsulin by trypsin and carboxypeptidase B. The major degradation by-product, A21-desamido insulin, is made by the detachment of the amine group of N_{21} in the A chain. Des-threonine (B30) insulin, arginyl-A0 insulin, and diarginyl-B31/B32 insulin are also made during the enzyme reaction [7, 15]. The substantial amount of insulin derivatives in the enzymatic conversion process could be a main obstacle for the commercialization of recombinant human insulin production [19].

In this study, the enzymatic modification of human proinsulin was carried out in the presence of a reducing agent, β-mercaptoethanol, which is used mainly in the refolding process of recombinant insulin. The second major derivative of human insulin was purified and characterized. To minimize the amount of the derivative, the effects of hydrogen peroxide on the conversion of human preproinsulin to insulin were explored in the enzyme reaction of trypsin and carboxypeptidase B. Additionally, recombinant human preproinsulin expressed in *E. coli* was subjected to enzymatic modification for the confirmation of the hydrogen peroxide effect.

Materials and Methods

Reagents

Human proinsulin with 98% purity and trypsin were purchased from Sigma Co. (St. Louis, MO, U.S.A.). Carboxypeptidase B was bought from Worthington Biochemical Co. (Lakewood, NJ, U.S.A.). Hydrogen peroxide, β-mercaptoethanol, and other chemical compounds were obtained from Sigma Co.

Enzymatic Modification of Human Proinsulin

The stock solution of 0.5 g/l human proinsulin was prepared by resolving it in 20 mM Tris buffer (pH 7.5) before use. A mixture of the enzyme reaction with or without 1 mM β-mercaptoethanol was composed of 0.5 g/l human proinsulin, 4.5 units of trypsin and 2.0 units of carboxypeptidase B in 20 mM Tris buffer (pH 7.5). After the incubation of the reaction mixture at 15°C for 16 h, the mixture was subjected to the analysis of human insulin and its derivatives by HPLC. In the case of hydrogen peroxide addition, the same enzyme reaction mixtures containing 1 mM to 100 mM hydrogen peroxide were incubated at 15°C for 16 h or 25°C for 6 h. A mixture containing 1 mM β-mercaptoethanol and 2 mM hydrogen peroxide was incubated at 25°C and pH 7.5 for 140 min and analyzed by HPLC.

Purification and Identification of Impurity 3

Impurity 3, a major derivative of human insulin except for the des-threonine human insulin, was purified by a cation-exchange chromatography with High S (Bio-rad, Hercules, CA, U.S.A.) and a reverse-phase chromatography with Kromasil C8 resin (Eka Chemicals AB, Göteborg, Sweden). The purified human insulin derivative was analyzed by matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry using a Voyager-DE Biospectrometry Workstation (Applied Biosystems, Foster City, CA, U.S.A.) located at the National Center for Inter-University Research Facility (Seoul, Korea). The N-terminal sequencing of the derivative was carried out at the Korea Basic Science Institute (Daejeon, Korea). Both analyses followed the default analysis parameters.

Expression, Purification, and Enzymatic Modification of Human Preproinsulin Expressed in Recombinant *E. coli*

Fed-batch fermentation of recombinant *E. coli* JM109/ pPT-MRpi expressing human preproinsulin under the control of the *P2* promoter [12] was carried out in a 300-l fermentor (B. Braun Biotech International, Germany) containing a minimal medium with 6 g/l glucose at 37°C. After the complete consumption of initial glucose, a mixture of 600 g/l glucose and 350 g/l yeast extract was fed into the bioreactor and the feed rate was controlled to maintain a 0.5 g/l glucose concentration in the culture broth, as described previously [17]. The culture broth at OD 58.2 was harvested by centrifugation at 12,000 rpm and 4°C for 20 min. The cell pellets were resuspended in buffer solution [5% sucrose, 0.1 M Tris,

50 mM EDTA, and 0.2 M sodium chloride (pH 7.9)]. The resuspended cells were lysed by using a homogenizer (Rannie, Copenhagen, Denmark). After centrifugation of the cell lysate, solid precipitates containing inclusion bodies of human preproinsulin were collected for unfolding and refolding processes. After washing the solid particles with a washing solution [0.1 M Tris, 1 mM EDTA, 1% Triton X-100, 0.02% lysozyme, and 2 M urea (pH 7.0)], the particles were mixed and solubilized with a solubilization buffer solution [4 M urea and 10 mM glycine (pH 10.6)]. The solution containing solubilized inclusion bodies was diluted with a refolding buffer solution [0.6 M urea and 10 mM glycine (pH 10.6)], of which the final concentration was 1.5 g/l. After addition of 0.3 mM β-mercaptoethanol into the diluted solution, the solution was stirred gently and incubated for 16 h at 15°C. The acidity of the solution was adjusted to pH 5.5 with 5 N HCl prior to centrifugation at 12,000 rpm and 4°C for 20 min. The refolded preproinsulin present in the supernatant was collected and used as a substrate for the same enzymatic modification at 15°C for 16 h. All samples of preproinsulin were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis as the standard method. Enzymes of 0.45 unit trypsin and 0.2 unit carboxypeptidase B per 0.5 mg of the refolded preproinsulin were added into the enzyme reaction mixture.

Conditions for High-Performance Liquid Chromatography

Human insulin, its derivatives, and chemicals formed by mixing hydrogen peroxide with β-mercaptoethanol were analyzed by a

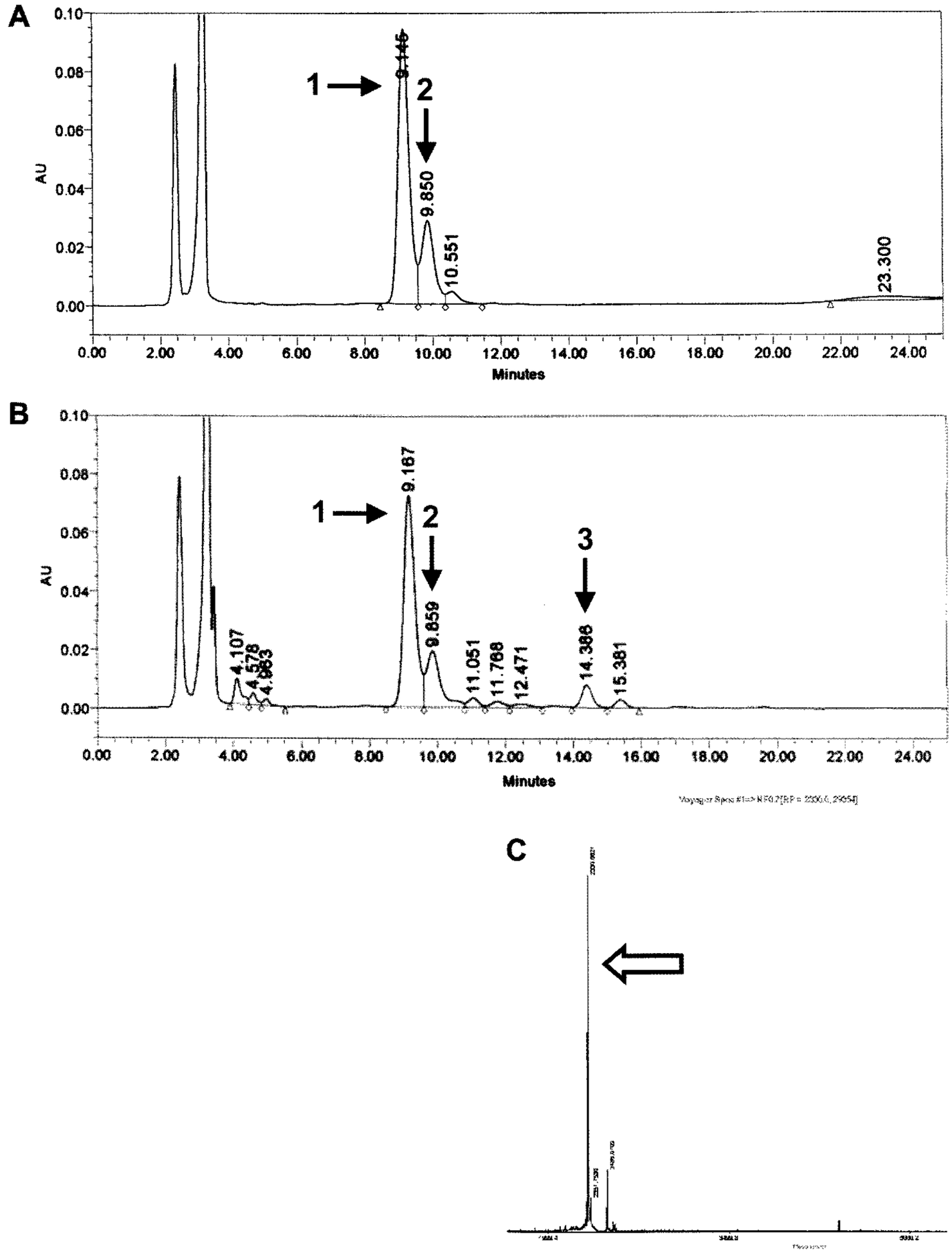

Fig. 2. HPLC diagrams for the analysis of human proinsulin digested by trypsin and carboxypeptidase B in the absence (**A**) and presence (**B**) of β-mercaptoethanol. Arrows indicate human insulin (1), des-threonine insulin (2), and impurity 3 (3). Purified impurity 3 was analyzed by MALDI-TOF mass spectrometry (**C**) and the arrow with white background shows the mass peak of impurity 3.

HPLC system (Alliance 2695; Waters, Milford, MA, U.S.A.) equipped with a Protein & Peptide C18 analytical column (250 mm×4.6 mm i.d., particle size 5 μm; Vydac, U.S.A.) heated at 40°C. The mobile phase consisted of 200 mM aqueous anhydrous sodium sulfate-acetonitrile (74:26, v/v) and flowed at 1 ml/min of flow rate. After injection of 20 μl of samples, absorbance at 214 nm of wavelength was monitored by a UV detector (Waters).

Results and Discussion

Effects of β-Mercaptoethanol on Enzymatic Modification of Human Proinsulin

Enzymatic modification of human proinsulin by proteases frequently caused the formation of its inactive derivatives. In some cases, their characteristics were thoroughly identified [15]. In a commercial process for human insulin manufacturing, β-mercaptoethanol was used as a reducing agent for the refolding of proinsulin inclusion body expressed in recombinant *E. coli* before the enzymatic modification of proinsulin. Generally, it was difficult to remove β-mercaptoethanol completely after the refolding process. Even if the complete removal of β-mercaptoethanol is available, additional steps and corresponding operation cost should be considered. The effects of β-mercaptoethanol on the formation of human insulin derivatives were investigated in the enzymatic modification of commercial human proinsulin by trypsin and carboxypeptidase B. The reaction samples without or with the treatment of 0.3 mM β-mercaptoethanol were analyzed by HPLC (Fig. 2). Addition of β-mercaptoethanol induced the formation of human insulin derivatives much more and hence reduced the conversion yield of human proinsulin to insulin by 33% compared with the case without β-mercaptoethanol. Among the various derivatives shown in Fig. 2B, a peak at 14.4 min of retention time was chosen and named impurity 3. Impurity 3 was the second major derivative found only in the presence of β-mercaptoethanol. It occupied 2% of the total amount of human insulin and its derivatives. To prevent the formation of impurity 3, a simple method would be developed by the addition of hydrogen peroxide.

Purification and Identification of Impurity 3

For the characterization of impurity 3, the impurity 3 with 98% of purity was purified by cation-exchange and reverse-phase chromatographies. Mass spectrometric analysis of the purified impurity 3 using MALDI-TOF mass spectrometry resulted in 2330.06 Da of molecular mass (Fig. 2C). Amino-terminal sequencing elucidated that the amino acid sequences of impurity 3 were identical to that of the human insulin B chain fragment from Phe_1 to Glu_{21}. For the mechanism of impurity 3 formation, a hypothesis is suggested that the cleavage of disulfide bonds by β-

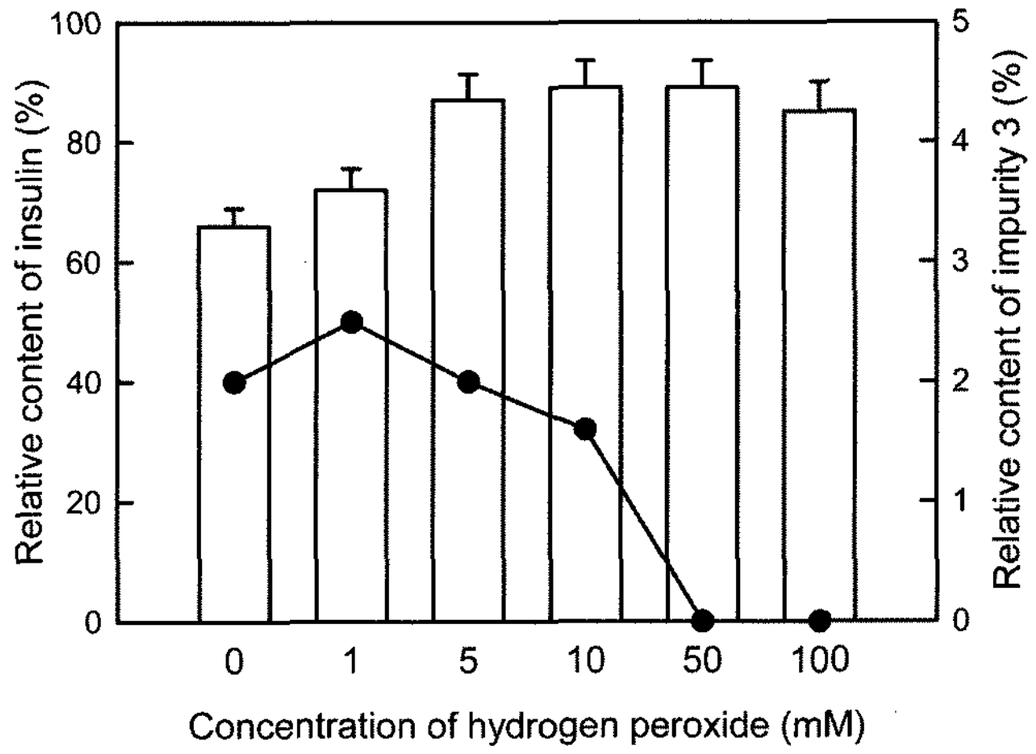

Fig. 3. Effects of hydrogen peroxide on the production of human insulin and impurity 3.
The enzymatic modification of human proinsulin by trypsin and carboxypeptidase B was performed for 16 h at 15°C in the presence of β-mercaptoethanol. The white bars indicate the relative amount of human insulin and the closed circle line graph shows the relative content of impurity 3.

mercaptoethanol allows the destruction of the tertiary structure of human proinsulin, and the peptide bond between Arg_{22} and Gly_{23} in the human insulin B chain is cleaved by trypsin, and Arg_{22} is successively removed by carboxypeptidase B. Finally, impurity 3, identical to the peptide fragment of the human insulin B chain from Phe_1 to Glu_{21}, is produced in the enzymatic modification process under β-mercaptoethanol addition. The addition of β-mercaptoethanol might facilitate the trypsin reaction on Arg_{22}, Lys_{29}, Arg_{32}, and Lys_{62} of the proinsulin, and hence undesirable by-products as well as impurity 3 are produced.

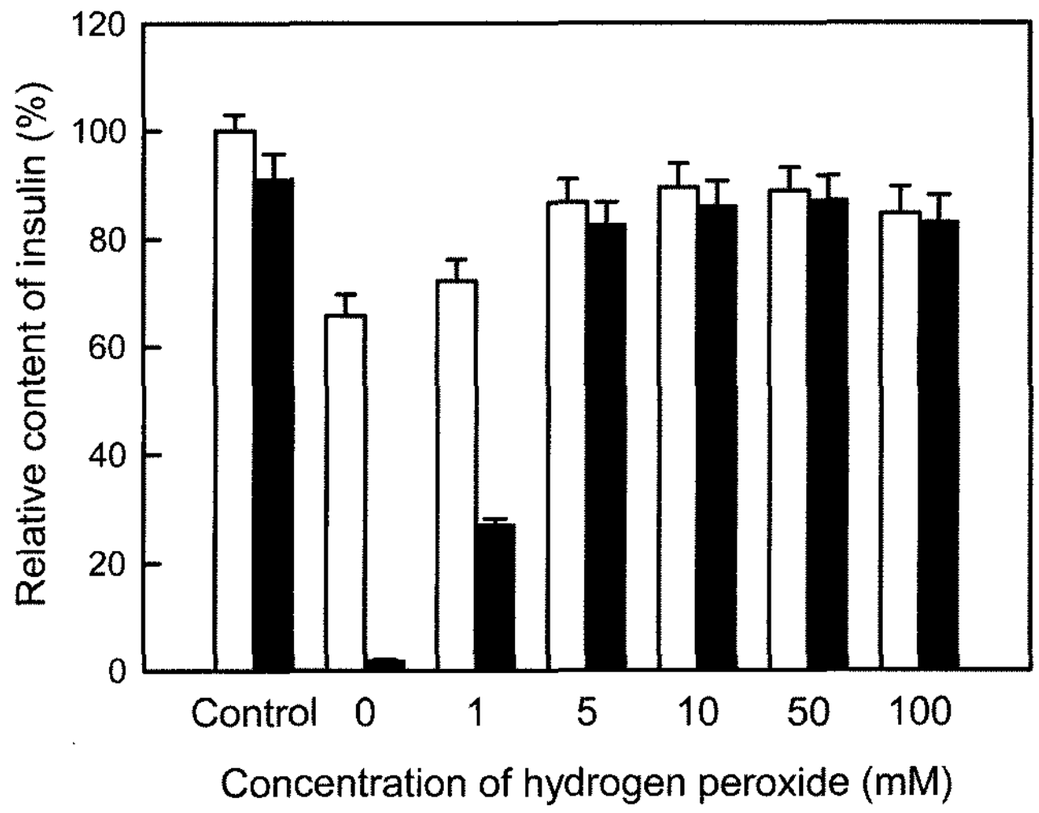

Fig. 4. Effects of hydrogen peroxide on the stability of converted human insulin in the enzyme reaction at 25°C.
The amount of converted human insulin was measured after 1 h (white bar) and 6 h (black bar) of reaction.

Effects of Hydrogen Peroxide on Enzymatic Conversion of Proinsulin

β-Mercaptoethanol increased the content of inactive insulin derivatives including impurity 3 in the enzymatic modification of human proinsulin. It caused the reduction of insulin conversion yield, and hence additional cost needs to be paid for obtaining active human insulin with high purity. To minimize the formation of impurity 3 in the presence of β-mercaptoethanol, hydrogen peroxide was added into the reaction mixture containing trypsin, carboxypeptidase B, and commercially available human proinsulin. After 16 h of incubation at 15°C, the contents of human insulin and impurity 3 were determined by HPLC and are depicted in Fig. 3. Without hydrogen peroxide, 66% of proinsulin was converted to active insulin and 2% accumulated as impurity 3. Along with an increase in hydrogen peroxide concentrations, the content of impurity 3 was dramatically reduced and the peak of impurity 3 in the HPLC diagram was not observed withe over 50 mM hydrogen peroxide. Concomitantly, the conversion ratio of proinsulin into insulin increased from 72% to 89%. When higher than 50 mM concentrations of hydrogen peroxide were added, an oxidized form of human insulin appeared (data not shown). Considering the conversion yield, impurity 3 content, and additional cost, the optimal concentration of hydrogen peroxide was determined at 10 mM.

Effects of Hydrogen Peroxide on Human Insulin Stability

To investigate the effects of hydrogen peroxide on the stability of converted human insulin, the content of converted human insulin was monitored after 1 and 6 h of enzyme reaction at 25°C (Fig. 4). As a control, the enzyme reaction without β-mercaptoethanol and hydrogen peroxide showed only 9% of reduction of converted human insulin content after 6 h. In the enzyme reaction with 1 mM β-mercaptoethanol and without hydrogen peroxide, about 97% of converted human insulin was degraded after 6 h of reaction. The stability of converted human insulin was enhanced with an increase in hydrogen peroxide concentration. At over 5 mM hydrogen peroxide concentration, 83–87% of converted human insulin remained stable for 6 h. The mechanism of the reduction of impurity 3 content and enhancement of insulin stability by hydrogen peroxide addition is not clear. However, HPLC analysis of the

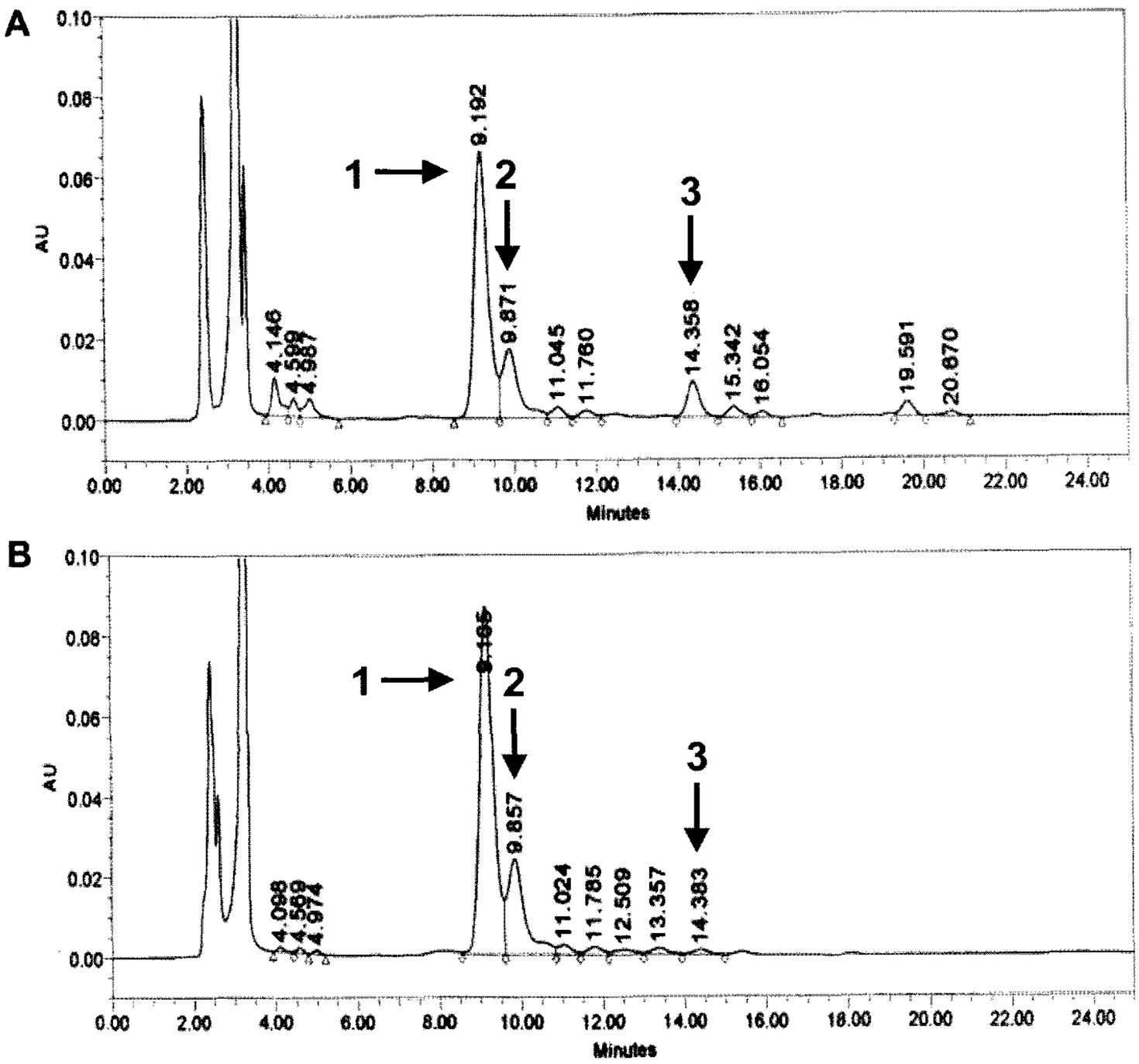

Fig. 5. Reverse-phase HPLC diagrams for the analysis of the enzymatic conversion of human preproinsulin expressed in recombinant *E. coli*.

In the absence (**A**) and presence (**B**) of hydrogen peroxide, the enzymatic modification was carried out in the reaction mixture containing trypsin, carboxypeptidase B, and β-mercaptoethanol. Numbers besides arrows indicate converted human insulin (1), des-threonine human insulin (2), and impurity 3 (3).

mixture containing 1 mM β-mercaptoethanol and 2 mM hydrogen peroxide and incubated at room temperature for 140 min showed an unknown peak that was enlarged with the reduction of β-mercaptoethanol and hydrogen peroxide peaks (data not shown). This new compound is not identified yet. Considering that the addition of hydrogen peroxide reduced the impurity content and enhanced the human insulin stability in the presence of β-mercaptoethanol, hydrogen peroxide seemed to transform β-mercaptoethanol to the new compound and thus diminish the negative effects of β-mercaptoethanol on the enzymatic modification of human proinsulin.

Application for Enzymatic Modification of Recombinant Human Preproinsulin

In order to verify the positive effects of hydrogen peroxide on the commercial process, purified and refolded human preproinsulin expressed in recombinant *E. coli* as a form of inclusion body was used for its enzymatic modification in the presence of β-mercaptoethanol. SDS-PAGE analysis of the refolded preproinsulin resulted in over 98% purity (data not shown). HPLC analysis of the reaction solution without hydrogen peroxide showed moderate amounts of insulin derivatives including des-threonine human insulin and impurity 3 (Fig. 5A). By addition of hydrogen peroxide, the peaks of impurity 3 and some unknown derivatives disappeared or their amounts decreased considerably (Fig. 5B). Concomitantly, the amounts of converted human insulin and des-threonine insulin were enhanced. Two lysine and five arginine residues in preproinsulin enable a large number of its derivatives to be synthesized in the enzyme reaction catalyzed by trypsin and carboxypeptidase B.

Because the B chain of insulin contains two potential tryptic cleavage sites (Arg_{22} and Lys_{29}), determination of highly selective reaction conditions should be required in order to minimize a loss of human insulin [1]. After the unfolding and refolding processes of the recombinant human preproinsulin expressed as inclusion body, it is difficult to completely eliminate β-mercaptoethanol-causing impurity formation in human proinsulin modification. A cheap chemical, hydrogen peroxide, bound with β-mercaptoethanol and hence minimized the negative effects of β-mercaptoethanol on the enzymatic modification of human proinsulin. The simple addition of hydrogen peroxide allowed the development of an economic process for human insulin manufacturing without high-cost consuming steps. More researches will be carried out to focus on the optimization of the enzymatic process for active human insulin production.

Acknowledgments

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