A simple, economical method of converting gene expression products of insulin into recombinant insulin and its application*

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Abstract A method, by which the gene expression product of recombinant single chain insulin can be converted into insulin by directly digesting with trypsin, has been established. This method has been used in process of porcine insulin precursor (PIP), [B16Ala] PIP and [B26Ala]PIP into (desB30)insulin, (desB30)[B16Ala]insulin and (desB30)[B26Ala]insulin, respectively, and all of them retain full biological activity of that of their corresponding parent, recombinant human insulin, [B16Ala]insulin and [B26Ala]insulin. The results further demonstrate that the C-terminal residue of B chain is not necessary for insulin's biological activity. Compared with the method of transpeptidation, this method is simple, with a high yield, and avoids the use of organic reagents, and in comparison with the trypsin/carboxypeptidase method, it omits the use of carboxylpeptidase. Besides, (desB30)[B16Ala]insulin and (desB30)[B26Ala]insulin still remained without self-association property as that of their parents, which demonstrate that they are monomeric insulin. So they can be used for substituting for monomeric insulin, [B16Ala]insulin and [B26Ala]insulin, in clinical applications.

Keywords: insulin, (desB30)insulin, trypsin/carboxylpeptidase, transpeptidation, monomeric insulin.

Insulin is a protein hormone, which consists of two polypeptide chains (named A chain and B chain, respectively) linked by two inter-chain disulfide bonds. However, it is synthesized *in vivo* as a single polypeptide chain called proinsulin. Proinsulin will be processed into mature insulin (double chain form) by specific protein enzymes *in vivo* and released into blood[1]. Now the recombinant human insulin has been extensively used in clinical practice to substitute for the insulin from animal. The expression products of recombinant insulin genes are also single chain polypeptide, which must be processed *in vitro* to become mature insulin before applied in clinical practice.

Generally, there are two methods for the process of the expression products of recombinant insulin genes: one of them is the combined use of trypsin and carboxylpeptidase[2,3]; the other is trypsin transpeptidation[4,5].

The mature insulin molecules have two cleavage sites of trypsin located at B22Arg and B29Lys, respectively, which makes the process of expression products of insulin genes have two by-products (desB30)insulin and (desB23-30)insulin (DOI). In addition, the trypsin transpeptidation reaction has to be caried out in organic reagents and finally the protective group must be removed by triflouroacetic acid (TFA). All of these will directly impair the quantity and yield of recombinant insulin. Therefore, the current methods of processing recombinant insulin products need to be improved.

As early as the 1950s, Van Abeele et al.[6] reported that the treatment of insulin preparation with low concentration trypsin did not impair insulin's biological activity although it caused inactivation of glucagon mixed in the insulin preparation. Li et al.[7] later confirmed that the product of trypsin treatment is mainly (desB30)insulin under the condition that Van Abeele et al. adopted and demonstrated that the residue at the position of 30th of B chain was not necessary for insulin's biological activity. Masaki et al.[8] found Achromobacter protease, (Lys-C), can specifically cleavage the peptide bond at the Lys carboxyl terminal. In the research work, people usually use the enzyme to obtain (desB30)insulin[9,10]. Because (desB30)insulin retains full biological activity of insulin and considering the enzyme Lys-C is too expensive to be used in industry, we have established a method of directly using trypsin to process expression products of single chain insulin genes, by which re-

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combinant (desB30)insulin with full biological activity has been obtained. Compared with the two current methods used for processing expressed products of single chain recombinant insulin genes, our method is simple, does not use organic reagents, has a high yield, and low cost. It can be developed to produce recombinant insulin in industrial scale[11].

1 Material and methods

1.1 Material

PIP with sequence of porcine insulin B chain-Ala-Lys-A chain and its derivatives[B16Ala]PIP and [B26Ala]PIP were expressed and purified according to previous reports[12~14]; monomeric insulin, [B28Lys, B29Pro]insulin, was enzymatic semi-synthesized in our laboratory; trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated) was purchased from Sigma. Other reagents used were all of analytical grade.

1.2 Methods

1.2.1 Preparation of (desB30)insulin Purified PIP was dissolved in 100 mmol/L NH_4HCO_3, pH8.0, at a concentration of 10 mg/mL, to which trypsin solution (dissolved in 100 mmol/L NH_4HCO_3, pH8.0) at concentration of 1 mg/mL was added with a ratio of PIP to trypsin of 200:1. The digestion reaction was carried out at 25 ℃, overnight. (DesB30)insulin was purified using HPLC[11].

1.2.2 Preparation of (desB30)[B16Ala]insulin and (desB30)[B26Ala]insulin Purified [B16Ala]PIP and [B26Ala]PIP were dissolved in 100 mmol/L NH_4HCO_3, pH8.5, respectively. The digestion reaction was performed by adding trypsin to the solution with a ratio of protein to enzyme of 200:1 at 25 ℃. During the reaction, a small quantity of reaction mixture was taken out and the reaction was stopped at 0, 1 h, 2 h, 3 h and 4 h, respectively, by lowering pH to 2.0 with TFA. The digestion of the reaction mixture was checked by native polyacrylamide gel electrophoresis (native PAGE, pH8.3). (DesB30)[B16Ala]insulin and (desB30)[B26Ala]insulin were purified with HPLC.

PIP, [B16Ala]PIP and [B26Ala]PIP were also digested by Lys-C overnight under the same condition to obtain (desB30)insulin, (desB30)[B16Ala]insulin, and (desB30)[B26Ala]insulin, respectively, as controls of those obtained by trypsin digestion.

1.2.3 Biological activity assay of (desB30)insulin, (desB30)[B16Ala]insulin and (desB30)[B26Ala]insulin Receptor binding capacity of (desB30)insulin and its derivatives was determined using human placental membrane[15]. A semi-quantitative mouse convulsion method[15] was used to measure *in vivo* biological activity. Briefly, for each dosage, 5 ICR mice (fast, male, weighing 18 ~ 20 g, purchased from SIPPR/BK Ltd.) were injected with the products and put in a chamber of 35 ℃. Their responses to the insulins were observed and recorded.

1.2.4 Self-association assay of (desB30)[B16Ala]insulin and (desB30)[B26Ala]insulin The self-association property of (desB30)[B16Ala]insulin and (desB30)[B26Ala]insulin was determined by FPLC as described[14,16]. The conditions were as the follows: the chromatography column used was Superdex 75 (HR10/30) which was eluted by phosphate buffer at pH7 with a flow rate of 0.4 mL/min at room temperature; the samples at the concentration of 1.2 mg/mL (2×10^{-4} mol/L) were loaded onto the column and detected at wavelength of 280 nm. Recombinant human insulin and [B28Lys, B29Pro]insulin were used as positive and negative self-association control, respectively[14].

2 Results

2.1 (DesB30)insulin

After digested with trypsin two products were obtained for PIP contains two cleavage sites of trypsin. One of the products, (desB30)insulin, retained full biological activity of insulin, while the other one (DOI) lost almost all of biological activity. The native PAGE pattern of PIP digest and purified (desB30)insulin is shown in Fig. 1. It shows that in the PIP digest there are a lot of by-products of DOI, which might be produced by an over-digestion.

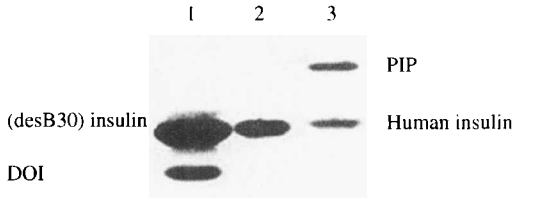

Fig. 1. Native PAGE pattern of trypsin digest of PIP and purified (desB30)insulin. 1, Trypsin digest; 2, HPLC purified (desB30) insulin; 3, control.

2.2 Preparation of (desB30)[B16Ala]insulin and (desB30)[B26Ala]insulin

In order to reduce the by-product DOI during the preparation of (desB30)[B16Ala]insulin and (desB30)[B26Ala]insulin, we carried out the experiments for obtaining optimum conditions of digestion (Fig.2). The results showed that the optimum conditions for the trypsin digestion of [B16Ala]PIP were in 100 mmol/L NH_4HCO_3, at pH8.5 with protein concentration of 10 mg/mL, the ratio of protein to enzyme was 200:1, and the digestion carried out at 25℃ for 1 ~ 2 h. The digest product (desB30)[B16Ala]insulin was purified by HPLC (Fig.3) with a yield of 70% ~ 80%. The (desB30)[B26Ala]insulin was obtained using the same procedure.

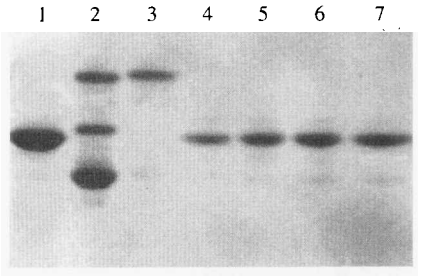

Fig. 2. PAGE pattern of digest of [B16Ala]PIP. 1, Digested PIP by Lys-C; 2, control (upper, PIP; middle, recombinant human insulin; lower, DOI); 3 ~ 7, trypsin-digested [B16Ala]PIP for 0, 1, 2, 3 and 4 h, respectively.

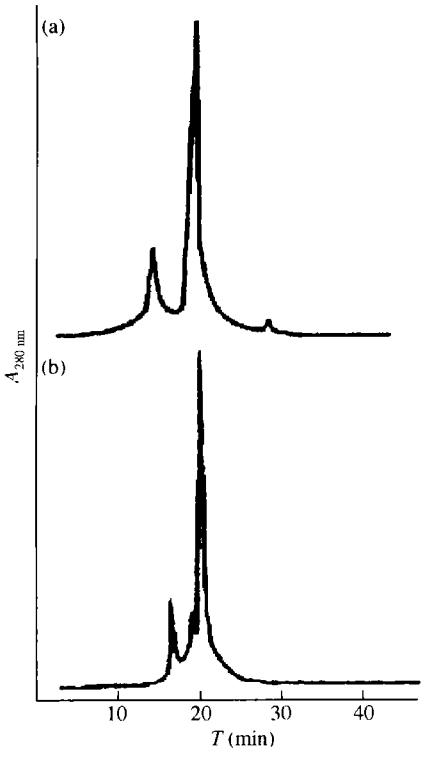

Fig. 3. HPLC profile of trypsin digests of [B16Ala]PIP and [B26Ala]PIP. (a) (DesB30)[B16Ala]insulin; (b) (desB30)[B26Ala]insulin.

The digestion of [B16Ala]PIP by Lys-C was also carried out overnight as a control of (desB30)[B16Ala]insulin obtained from the digestion of trypsin (Fig.2).

2.3 Characterization of (desB30)[B16Ala]insulin and (desB30)[B26Ala]insulin

PAGE pattern shows that both purified (desB30)[B16Ala]insulin and (desB30)[B26Ala]insulin obtained by HPLC are single band (Fig. 4). The molecular weight of them measured with electrospray mass spectrometry is listed in Table 1, which shows that measured values agree with the theoretical values.

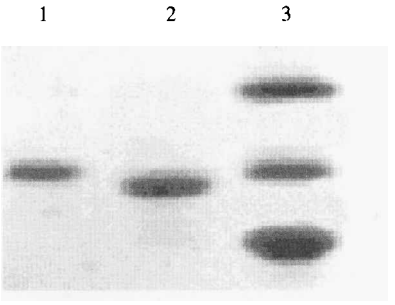

Fig. 4. PAGE pattern of purified (desB30)[B16Ala]insulin and (desB30)[B26Ala]insulin. 1, (desB30)[B16Ala]insulin; 2, (desB30)[B26Ala]insulin; 3, control (upper, PIP; middle, recombinant human insulin; lower, DOI).

Table 1. Molecular weight of (desB30)[B16Ala]insulin and (desB30)[B26Ala]insulin measured by electrospray mass spectrometry

Sample	Theoretical value	Measured value
(DesB30)[B16Ala]insulin	5614.5	5614.0
(DesB30)[B26Ala]insulin	5614.5	5613.4

2.4 Biological activity assay of (desB30)insulin, (desB30)[B16Ala]insulin and (desB30)[B26Ala]insulin

The receptor binding capacity of (desB30)insulin, (desB30)[B16Ala]insulin and (desB30)[B26Ala]insulin with the insulin receptor on human placental membrane, which was calculated by the dosage used to inhibit 50% of bound ^{125}I-insulin with the receptor, were respectively 100%, 17.9% and 63.1% of that of native porcine insulin (Fig. 5). Table 2 lists *in vivo* biological activity of (desB30)[B16Ala]insulin and (desB30)[B26Ala]insulin. When compared to their parents, recombinant human

insulin[3], [B16Ala]insulin[14] and [B26Ala]insulin[14], (desB30)insulin and its derivatives retained all the biological activity *in vitro* and *in vivo*.

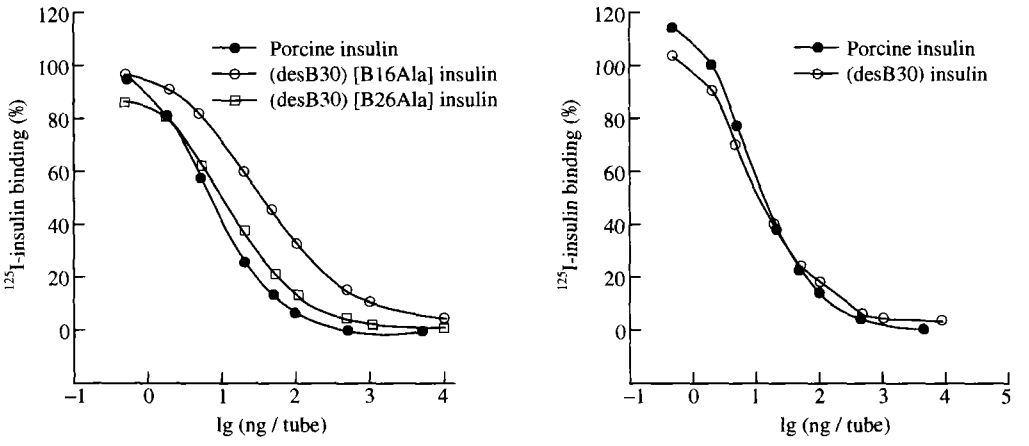

Fig. 5. Receptor binding activity of (desB30)insulin, (desB30)[B16Ala]insulin and (desB30)[B26Ala]insulin with insulin receptor on human placental membrane.

Table 2. *In vivo* biological activity of (desB30)[B16Ala]insulin and (desB30)[B26Ala]insulin measured with mouse convulsion method

	Dosage (μg)[a]			
	0.25	0.5	1	2
Porcine insulin	0/5	2/5	5/5	
(DesB30)[B16Ala]insulin	0/5	2/5	4/5	5/5
(DesB30)[B26Ala]insulin	0/5	2/5	5/5	

a) Denominator, number of mice injected; numerator, number of mice with convulsion occurred.

2.5 Determination of self-association of (desB30)[B16Ala]insulin and (desB30)[B26Ala]insulin

Self-association property of (desB30)[B16Ala]insulin and (desB30)[B26Ala]insulin was also measured using molecular sieve on FPLC since their parents are monomeric insulins[14]. [B28Lys, B29Pro]insulin and recombinant human insulin were used as the monomeric form and association form controls, respectively. Fig. 6 shows the elution profiles of the two derivatives and controls, which indicate that the retention time of (desB30)[B16Ala]insulin and (desB30)[B26Ala]insulin is longer than that of recombinant human insulin and the shape of peaks is symmetric like the monomeric insulin of [B28Lys, B29Pro]insulin. Therefore, the results demonstrate that (desB30)[B16Ala]insulin and (desB30)[B26Ala]insulin had lost self-association property like their parents and they are monomeric insulin.

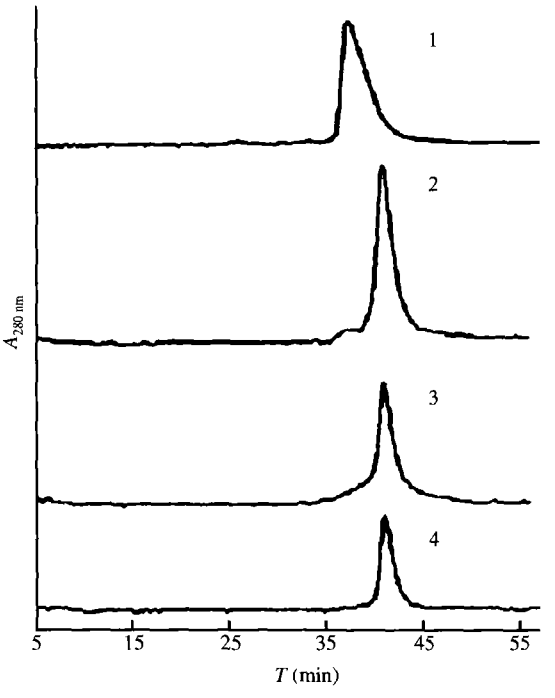

Fig. 6. Profile of FPLC showing retention time and the peaks. 1, recombinant human insulin; 2, [B28Lys, B29Pro]insulin; 3, (desB30)[B16Ala]insulin; 4, (desB30)[B26Ala]insulin.

3 Discussion

We have established a method of processing expressed recombinant PIP and its derivatives with full biological activity by a single step trypsin digest. The (desB30)[B16Ala]insulin and (desB30)[B26Ala]insulin obtained by this method are also monomeric insulin like their parents. The method has the advantages of (1) avoiding using organic reagents such as dimethylsulfoxid (DMSO), 1, 4-butanediol and O-tert-butyl-L-threonine tert-butyl ester acetate, etc. which have to be used in the trypsin transpeptidation[4,5]; (2) omitting the use of carboxylpeptidase

which is one of the main enzyme preparations in the method of trypsin/carboxylpeptidase[2,3]; (3) being simple, economic, and with a high yield. Therefore, the method can be used in industrial production on a large scale.

The results further confirmed that the 30th residue in the B-chain is not necessary for insulin's biological activity since (desB30)insulin, (desB30)[B16Ala]insulin and (desB30)[B26Ala]insulin all retain the full biological activity of that of their corresponding parents. In the view of structure, the residue at C-terminal of B-chain does not participate in the construction of secondary structure[17,18], so deletion of the residue will not affect insulin structure; in terms of biological function, the results together with previous reports[6,7,9,10] further demonstrate that the residue at the position B30 is not necessary for insulin's biological activity; in the view of physiology, deleting an unnecessary terminal residue from a protein will have smaller effect than mutanting a residue on an organic body. In addition, the (desB30)insulin may be one of the products of metabolism of native insulin. Therefore, (desB30)insulin can be accepted in clinical use.

Insulin is the most effective medicine for treatment of diabetes and has been used in clinical practice for more than 80 years, although it still has some flaws in the treatment. One of the problems is that insulin possesses the self-association property which makes insulin act not as quickly as required after injection since the active form of insulin is a monomer. It will cause the highest blood sugar content and the highest blood insulin content occurring at different time after meal, making diabetic patients suffer from many side effects (named diabetic syndrome) which has seriously imperiled health and life of patients[9]. In order to prevent the self-association of insulin, people have prepared several insulin analogs without self-association, called monomeric insulin, such as [B28Lys, B29Pro]in sulin[20], [B28Asp]insulin[21], [B16Ala]in sulin[13,14], and [B26Ala]insulin[13,14], etc. The (desB30)[B16Ala]insulin and (desB30)[B26Ala]insulin we obtained not only retain full biological activity of that of their corresponding parents but also have no self-association property, which therefore will be beneficial to the diabetes patients in the treatment.

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