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آموزش مهارت های کاربردی در تدوین و چاپ مقاله

اصول تنظیم قراردادها

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Kinetic Study of Trypsin Enzymatic Effect on Digestion of Refolded Recombinant Insulin by HPLC, Elisa, Non-Denaturing and Tris-Tricin PAGE Methods

Tahereh Sadeghcheh*, Atousa Aliahmadi, Ali Moradi, Maryam Omidi, Mehrnaz Azami, Mansour Abachi, Soroush Sardari, Mohammad Reza Jebelli, Ahmad Adeli, Fereidoun Mahboudi**

Biotechnology Research Center, Pasteur Institute of Iran
Email Address: mahboudi@institute.pasteure.ac.ir

Abstract

An analytical scheme for monitoring recombinant human insulin digestion is suggested. The scheme includes high Performance Liquid Chromatography, Non-Denaturing and Tris-Tricin PAGE and Elisa .The use of this scheme at all digestion steps provided optimization of certain parameters (conditions for fusion protein FP duration of cleavage by trypsin. The proposed scheme may be used for solving various problems in digestion of other recombinant proteins.

Introduction

Insulin is a hormone which maintains the blood glucose level at the normal range and is used to treat diabetes. Recombinant human pro-insulin can be produced in *E.coli* cells as inclusion bodies. (Petrieds, D. 1995)
Fusion protein (FP) isolated from inclusion bodies should be purified, reduced, refolded, isolated, and then Trypsinolysed. (Jonasson,, P. 1996, Lillei,H. 1998, Futami,J. 2000) With the treatment of trypsin on denatured FP, it could be converted to Arg-insulin. However, by controlling the amount of the enzyme, the reaction time, temperature, buffer concentration and the ratio of enzyme to substrate, the optimized reaction time for trypsin can be determined. (Sergeev,N.V.,2001)

Materials and Methods

Based on plasmid construction *E.coli* competent cell which donated by Institute of Biology, Pushchinov, Moscow transformation, fusion protein expression, isolation, sulfitolysis and purification of FP by DEAE Sepharose chromatography were done.(Jebelli, M.R., 2003 , Ghazi,I. 2003)

Refolding

The fractions after DEAE chromatography containing FP (protein concentration 5mg/ml in Glycin 50 mM, pH 10) were diluted up to final protein concentration 0.3mg/ml. The pH was adjusted to 10.5 with NaOH 0.2 M. The reaction was started at the desired temperature (4^0C was found to be optimal) by addition of redox agents (cystine 1.25mg and 2-ME 7.54 µl for every 177mg of protein) and the reaction mixture was incubated over night at 4^0C.

Enzymatic Cleavage by Trypsin

The buffer of folded protein FP (0.3mg/ml) was Glycin 50mM , pH 10 and it had to be changed to Tris-HCl 50 mM , pH 8 with using Centriprep (cut off 10 kDa) according to manufacturing instruction . This step repeated 3 times and final concentration of protein was 1mg/ml . Protein solution was pre-warmed at 37^0C for 5 min. and pH was adjusted at 8.0 again .The sample of protein was subjected to trypsin (Sigma T-800) cleavage at a ratio of 1:50 (trysin:FP , w/w).The sample was incubated at 37^0C for 90 minute. Sampling was done at different time intervals and the reaction was stopped by addition of PMSF to final concentration of 2mM.

Analytical Reversed Phase HPLC

It was performed on a Pharmacia LKB HPLC system (pump 245, column oven 2155, HPLC manager software Pharmacia LKB biotechnology, Uppsala, Sweden, c18 column) which has a good selectivity for analysis of Insulin. Elution was carried out at a flow rate of 0.8ml/min with a linear gradient of Acetonitrile (0-100% for 80 minute) in a 0.1% TFA at 45^0C. The concentration of each fraction was determined by Bradford method and all of samples were freeze-dried by CHIRIS ALPHA RVC Drier at -80^0C, under vacuums.

Tris-Tricin PAGE

Polypeptides with the weight lower than 12KD in normal SDS-PAGE with Tris-Glycin buffer system cannot be separate properly, even in high concentration gels, so the system of Tris - Tricin was selected as a better technique for insulin and proinsulin. C% of all resolving, middle and stacking gels was 3% but T% was 16.5%, 10%, and 4% respectively. 50 µg of every fraction was dissolved in dH_2O and mixed with sample buffer at a ratio of 1:1 and heated at 95^0C so they were applied on Tris-Tricin gel and were run at constant voltage of 100 mv for at least 4 hour.

Non-Denaturing PAGE

In another analytical method all digested samples with different reaction time were applied on 15% non-denaturing PAGE containing 10% Glycerol, without any reducing agents like SDS, 2ME or heating. The gel was run at the same condition of Tris-Tricin PAGE.

Elisa Assay

The assay was done by DIAPLUS Human Insulin Elisa Test Kit (Diaplus, Italy, Roma) for *invitro* diagnostic use. 10 µg of all of fractions in 50 µl $dH_2O$and also 50 µl of controls and calibrations in duplicated form at 20-25^0C were applied on micro plate wells, then 100 µl of biotinylated enzyme labeled antibody added to each well, after 120 minute incubation and washing 3 times with washing buffer, the color developed with TMB (tetramethyl benzidine) and the absorbance was read at 450 nm using a reference wavelength of 620-630 nm to minimize well imperfections in micro plate Elisa Reader (BW 50 BIOHIT).

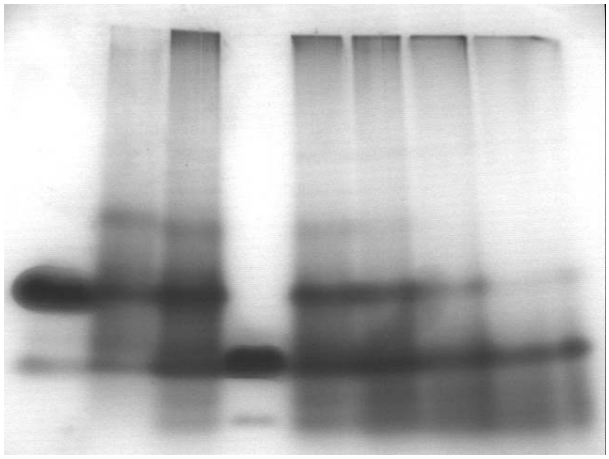
Results and Discussion

According to patterns of non-denaturing and Tris-tricin gels, it was obvious that continuation of enzymatic assay more than 15 minute was unnecessary and maximum efficiency of reaction was between 10 to 15 minute (figure) and also the results of Elisa showed the maximum OD at 450 nm, obtained from samples with 10 to 15 minute incubation time. These results were confirmed by patterns of HPLC chromatogram.

Separation of insulin-like Peptides is a difficult task, because of their slight difference in mass and charge, so analytical techniques such as reverse phase high performance liquid chromatography (RP-HPLC) is needed which exploit differences in molecular hydrophobicity.

All samples were analyzed with human insulin Elisa kit. Immunoenzymometric assay includes high affinity and specific antibodies with different and distinct epitope recognition in excess and native antigen. The enzyme activity in the antibody-bound fraction is directly proportional to the antigen concentration. The peptides resulted from trypsinolysis could be analyzed with Tris-Tricin and non-denaturing PAGE, because these kind of gels show better separation of small peptides.

1 2 3 4 5 6 7 8



1) Argenine-insulin
2) Before digestion
3) Digested sample after 10 min
4) Digested sample after 15 min
5) Insulin
6) Digested sample after 30 min
7) Digested sample after 60 min
8) Digested sample after 90 min

Figure: 15 % non-denaturing PAGE.

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