

Semisynthesis of human insulin: transpeptidation or coupling mechanism?

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Dedicated to Acad. Ivan Juchnovski on the occasion of his 80th birthday

The polypeptides and proteins were responsible for essentially all the activities of the biological world. Insulin as a polypeptide hormone involved in the regulation of blood sugar, and is an important objective of modern biomedical research. Herein, we report the semisynthetic transformation of porcine into human insulin. A simple HPLC method and different analysis were used to obtain the evidence for the detailed mechanism of trypsin catalyzed reaction of semisynthetic transformation of animal insulin.

Key words: porcine insulin; human insulin; trypsin; enzyme transformation; HPLC

INTRODUCTION

Many publications reflect of important role of the insulin in the development of peptide chemistry, pharmacology, cell signaling and structural biology. These discoveries have provided a steadily improved quantity and quality of life for those afflicted with diabetes [1].

The interest in the synthesis of insulin and insulin analogues by chemical methods has recently increased owing to improvements in reagents, resins and methodology [2, 3]. Two methodologies were validated as effective methods of insulin synthesis: chemical/semisynthetic synthesis or using recombinant DNA-based technology.

Difference between human and porcine insulin is at the B- chain (Thr instead of Ala) of the polypeptide. The mechanism of semisynthetic transformation of porcine into human insulin is not clear till now.

The conclusion of Rose et al [4] is that the transformation occurs, by a mechanism involving hydrolysis followed by coupling, and not by direct transpeptidation as has been previously found the case for another similar systems [5].

Where is the true, in the mechanism involving hydrolysis followed by coupling, or direct transpeptidation?

The aim of this paper is to find the evidence for the mechanism of enzyme catalyzed transformation of animal into human insulin.

EXPERIMENTAL

Materials and Methods

Trypsin LKB (TPCK), trifluoroacetic acid (TFA), 1, 4–butanediol 1, 5–pentanediol, 1, 6–hexanediol, H-Thr-OMe, porcine and human insulin reference standard (United States Pharmacopeia) were obtained from Sigma - Aldrich (Germany). The enzyme activity of trypsin was determined according [6]. HCl, CH₃COOH, CH₃COCH₃, CaCO₃, NaOH, CH₃CN – gradient grade for HPLC were obtained from Merck (Germany). Monocomponent porcine insulin № 21002 (Sopharma) was used in reactions of enzymatic transformation.

Amino acid analyses

The amino acid content of insulin samples were calculated by using a BIOTRONIK automatic analyzer model 6001 after 24 h, 48 h and 72 h of hydrolysis in 6 M HCl in evacuated sealed tubes at 110° C.

HPLC analysis

The samples were chromatographed on HPLC Shimadzu Model LC 2010 A with UV detector. Zorbax -300 SB-C₈ column, 4,6 x 250 mm, 5 μm and guard column Zorbax -300 SB-C₈ column, 4,6 x 12,5 mm, 5 μm, with a 28 -50 % linear gradient

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of 0,1% TFA/water - 0,1% CH₃CN /water for 55 minutes were used for monitoring of the studied reactions. The flow rate was 1 ml/min and the absorbance was monitored at 214 nm.

RESULTS AND DISCUSSION

Over the many years semisynthesis has been the predominant synthetic method in the preparation of human insulin or insulin analogs [7-14]. One of the strategies is to use targeted enzymatic, or chemical degradation of native insulin as a starting point for synthesis of the desired analog.

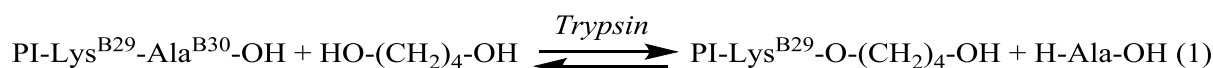
For the semisynthetic transformation of porcine insulin into insulin of human sequence three extreme mechanistic cases may be considered [1]:

(i) the reaction proceeds via aminolysis of the acyl-enzyme intermediate (i.e. via transpeptidation) without prior hydrolysis to des-Ala-B30-insulin;

(ii) porcine insulin is hydrolysed to des-Ala-B30-insulin, which then undergoes immediate coupling to give product;

(iii) porcine insulin, and there after acyl-enzyme intermediate, is hydrolysed reversibly to des-Ala-B30-insulin.

In this paper the trypsin (T) catalyzed reaction of transformation of porcine into human insulin was carry out in the mixed organic solvent N,N-dimethylacetamide and differend diols:1,4-butanediol, 1,5-pentanediol, 1,6- hexanediol in the ratio 1:1 (v/v), in the presence of H-Thr-OMe, at 12-15°C. The best result was obtained by using 1,4-butanediol. The experimental dates in the absence of the H-Thr-OMe showed that 1,4-butanediol reacts as a nucleophile in the formation of des-Ala-insulin-4 hydroxy-butyl-ester and free alanine was released according the equation (1):



Formation of the porcine des-Ala-insulin-butyl ester was monitored by HPLC analysis of the reaction components Fig. 1. Such kind of reaction of porcine insulin in the presence of alcohol is experimentally registered for the first time.

Kinetic of the reaction was followed at 214 nm by measurements of the concentration changes of the starting porcine insulin and formation of ester of des-Ala-insulin Fig. 2. Amino acid analysis of porcine des-Ala-insulin (theoretical values in brackets) give: Lys 0,98 (1), His 1,8 (2), Arg 0,87 (1), Asp 3,12 (3), Thr 2,03 (2), Ser 2,79 (3), Glu 6,87 (7), Pro 1,19 (1), Gly 4,15 (4), Ala 1,16 (1), CySO₃H 5,68 (6), Val 3,73 (4), Ile 1,68 (2), Leu 6,33 (6), Tyr 3,78 (4) and Phe 3,21 (3).These data cofirms the absence of one alanine in molecule of the porsine insulin.

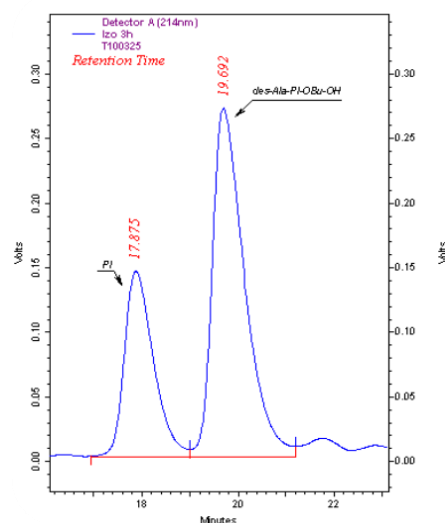


Fig.1. Representative HPLC chromatogram of the formation of butyl ester of porcine des-Ala-insulin, PI-porcine insulin; column Zorbax -300 SB-C₈ 4,6 x 250 mm, linear gradient for 55 min, 0,1% TFA/water-0,1% CH₃CN/water , flow rate 1 ml/min, detection at 214 nm.

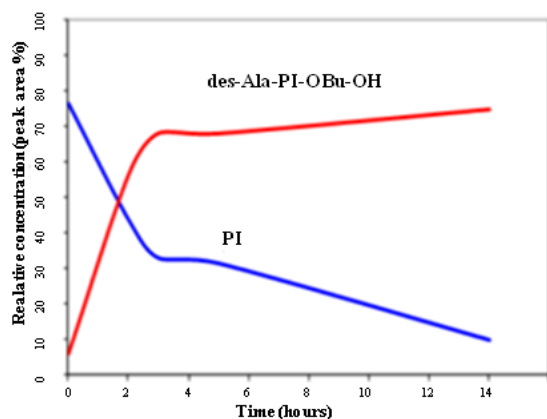
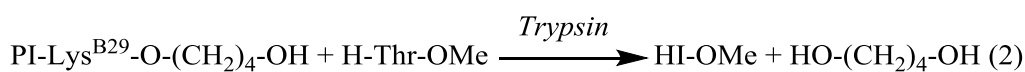


Fig.2. Time dependence of the concentration changes in trypsin catalyzed reaction of the hydrolysis of porcine insulin in the presence of 1,4-butanediol. Reaction conditions: 100 mg porcine insulin dissolved in 0.5 ml 10 M CH₃COOH, 2 ml N, N-dimethylacetamide / 1,4-butanediol (1:1 v/v), 10 mg (4, 2 x 10⁻⁷ mol) trypsin, 12°C.



When the reaction was carry out in the presence of H-Thr-OMe equation (2), we observed very fast disappearance of the kinetically controlled obtained des-Ala-insulin-4 hydroxy-butyl-esteris Fig 3.

This is a key factor in the enzymatic catalyzed synthesis of human insulin, leading to short reaction time and low side reaction products. The 1, 4-butanediol only accelerate the reaction and is released in the end without of change. We can conclude that it reacts as a catalyst of the transformation reaction. The yield of human insulin methyl ester varies from 68 % to 82% depending on the experimental conditions, as amount of enzyme, ratio of reagents or solvents and temperature.

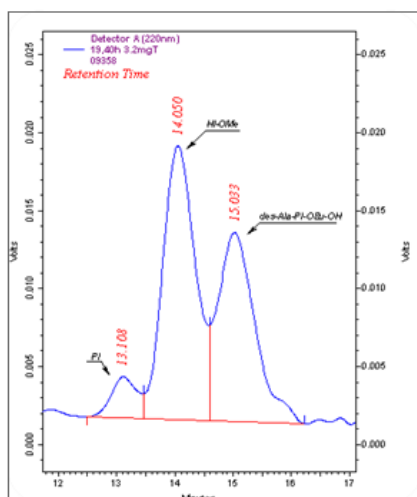


Fig.3. HPLC chromatogram of the formation of human insulin methyl ester from porcine insulin in the trypsin catalyzed reaction. Reaction conditions:100 mg porcine insulin dissolved in 0.5 ml 10 M CH₃COOH, 2 ml N,N – dimethylacetamide / 1,4- butanediol (1:1 v/v), 0.343 g (2,57x10⁻³ mol) H-Thr-OMe, 10 mg (4 x 10⁻⁷ mol) trypsin, 12°C.

CONCLUSION

The results in this study point unambiguously that enzymatic catalyzed semisynthesis of human insulin pass under coupling mechanism. As to our knowledge such direct involve of alcohol in formation of reactive intermediate compound, accelerating the reaction was registered for the first time experimentally.

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REFERENCES

1. D. R. Owens, B. Zinman, G. B. Bolli, *The Lancet*, **358**, 739 (2001).
2. A. N. Zaykov, J. P. Mayer, R. D. DiMarchi, *Nat. Rev. Drug Discov.*, **15**, 425 (2016).
3. A. N. Zaykov, J. P. Mayer, V. M. Gelfanov, R. D. DiMarchi, *ACS Chem. Biol.*, **9**, 683 (2014).
4. K. Rose, J. Gladstone, R. E. Offord, *Biochem. J.*, **220**, 189 (1984).
5. K. Rose, R. Stöcklin, L. A. Savoy, P. O. Regamey, R. E Offord, P. Vuagnat, J. Markussen, *Protein Eng.*, **4**, 409 (1991).
6. H. Bergmeyer, K. Gawehn, M. Grassl, *Methods of Enzymatic Analysis*, H. U. Bergmeyer (ed.), Academic Press, New York, 197, p. 4515.
7. M. A. Rittenberg, *Science*, **177**, 623 (1972).
8. K. Inouye, K. Watanabe, K. Morihara, Y. Tochino, T. Kanaya, J. Emura, S. Sakakibara, *J. Am. Chem. Soc.*, **101**, 751 (1979).

9. K. Morihara, T. Oka, H. Tsuzuki, *Nature*, **280**, 412 (1979).
10. K. Morihara, T. Oka, H. Tsuzuki, Washington, DC, *U.S. Patent* 4 511 505. (1985).
11. U. Yoshihiko, K. Morihara, *Biotech. Bioeng.*, **33**, 126 (1989).
12. K. Morihara, *J. Mol. Recognit.*, **3**, 181 (1990).
13. J. G. Ding, D. F. Cui, Y. S. Zhang, *Acta Bioch. Bioph. Sin.*, **35**, 215 (2003).
14. J. P. Mayer, F. Zhang, R. D. DiMarchi, *Biopolymers* **88**, 687 (2007).

СЕМИСИНТЕЗ НА ЧОВЕШКИ ИНСУЛИН: ТРАНСПЕПТИДИРАНЕ ИЛИ КОНДЕНЗАЦИОНЕН МЕХАНИЗЪМ?

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(Резюме)

Полипептидите и протеините имат голямо значение за почти всички процеси в биологичния свят. Инсулинът като полипептиден хормон, участващ в регулирането на кръвната захар, е обект на интензивни изследвания в съвременната биомедицина. Цел на това изследване е да се установи детайлният механизъм по който протича ензимно катализираната трансформация на свински в човешки инсулин. Високо-ефективна течна хроматография, (ВЕТХ) аминокиселинен анализ (АА) и кинетични изследвания бяха използвани за определяне на механизма по който се осъществява катализирана от трипсин реакция на трансформация на животински в човешки инсулин. Установено беше, че при избраните от нас експериментални условия, реакцията протича по кондензационен механизъм.