Volume 212, number 2, 307–312 FEB 04459 February 1987

Secretion of human insulin by a transformed yeast cell

Lars Thim, Mogens T. Hansen and Anders R. Sørensen

Novo Research Institute, Novo Allé, DK-2880 Bagsvaerd, Denmark

Received 18 December 1986

A yeast expression plasmid encoding a mini-proinsulin molecule was constructed and transformed into *Saccharomyces cerevisiae*. The plasmid encoded the sequence: B-Arg-Arg-Leu-Gln-Lys-Arg-A in which B represents the B-chain (30 amino acid residues) and A represents the A-chain (21 amino acid residues) of human insulin. The secreted peptides were shown to be a mixture of human insulin and des(B-30)human insulin. Thus, correct disulphide bridges can be established in proinsulin-like molecules devoid of a normal C-peptide region. Furthermore, the specificity of the yeast processing enzymes is so similar to the proinsulin converting enzymes in the human pancreatic β-cell that it allows the processing of the mini-proinsulin to insulin.

Proinsulin; C-peptide; Processing; Dibasic sequence; Hormone precursor

1. INTRODUCTION

Secreted peptide pheromones are triggers which are necessary to initiate mating between haploid cells of *Saccharomyces cerevisiae* [1]. One pheromone, the α-factor, is a peptide of 13 amino acid residues [2] which is synthesized in the yeast cells as a high molecular mass precursor [3]. The sequencing of the mating factor α_1 precursor gene (MFα1) revealed that the α-factor is present in four repeats separated by spacer peptides with the sequences of Lys-Arg-(Glu-Ala)$_3$ or Lys-Arg-Glu-Ala-Asp-Ala-Glu-Ala [3]. The posttranslational processing of the precursor includes trypsin-like cleavages at Lys-Arg sequences and dipeptidyl aminopeptidase-like cleavages for the removal of the Glu-Ala and Asp-Ala sequences [4].

In the pancreatic β-cell of mammals a similar enzyme system is responsible for the posttranslational processing of proinsulin to insulin (for review see Steiner et al. [5]). We have previously studied the secretion and processing in yeast of a series of insulin precursors containing one or two dibasic sequences [6]. These studies revealed that proinsulin is rapidly cleaved at one or both dibasic sequences and that these cleavages to a large extent occur before disulphide bond formation. However, when the C-peptide of 31 amino acid residues was replaced by a spacer peptide of two amino acid residues (Leu-Gln), a higher amount of insulin-like peptides was secreted [6]. The present study was undertaken in order to determine the exact structure of the insulin-like peptides secreted by a transformed yeast encoding this mini-proinsulin molecule. From the structural studies the pattern of posttranslational processing could be deduced and the similarity between the yeast processing enzyme system and the proinsulin-converting enzyme system could be further elucidated.

Correspondence address: L. Thim, Novo Research Institute, DK-2880 Bagsvaerd, Denmark

2. MATERIALS AND METHODS

2.1. *Plasmid construction*

The yeast expression plasmid encoding the mini-proinsulin sequence has been described in detail elsewhere [6]. Briefly the gene encoding the first 85

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

amino acid residues of the yeast mating factor α_1 (MFα1) leader sequence [4] was fused to the mini-proinsulin encoding gene in the configuration: MFα1(1–83)-Lys-Arg-B-Arg-Arg-Leu-Gln-Lys-Arg-A in which B and A represent the B-chain and A-chain of human insulin. This construction was inserted between the promoter and the transcription termination regions of the triose phosphate isomerase gene of *S. cerevisiae* and the entire expression unit was inserted in plasmid CPOT [7], which was transformed into a triose phosphate isomerase deficient strain of *S. cerevisiae* [6].

2.2. *Peptide purification*

The transformed yeast strain was grown on a YPD medium [8] to an optical density at 660 nm of 9.0 (37°C, 72 h). The yeast culture was centrifuged and 150 ml of ethanol was added to 1.5 l of yeast supernatant. The solution was passed through a column (2.6 × 4.0 cm) of LiChroprep RP-18, 25–40 μm (Merck) at a flow rate of 100 ml/h. The column was washed with 50 ml of 0.1 M NaCl followed by 50 ml of water and the peptide material was eluted with 60% (v/v) ethanol in 50 mM ammonium hydrogencarbonate. The eluate was concentrated to 5 ml by vacuum centrifugation to remove the ethanol, and the volume was adjusted to 10 ml by the addition of 5 ml of 50 mM Hepes buffer (pH 7.4). The sample was applied to an anti-insulin Sepharose column (2.5 × 4.5 cm) equilibrated in 25 mM Hepes buffer (pH 7.4). Anti-insulin Sepharose was prepared by coupling of monoclonal insulin antibody (MCA OXI 001, Novo Industri A/S) to CNBr-activated Sepharose (Pharmacia) as described by the manufacturer (5 mg/ml gel). The column was allowed to stand for 30 min at room temperature and washed with 40 ml of 25 mM Hepes buffer (pH 7.4). Peptide material was eluted with 10% (v/v) acetic acid and the pH of the eluate was adjusted to 4.0 with 2 N NaOH. The peptide material was finally purified by HPLC on a Macherey-Nagel 5 μm Nucleosil RP C-18 column (4 × 200 mm) equilibrated and isocratically eluted with 33 mM ammonium sulphate, 1.5 mM sulphuric acid containing 29.4% (v/v) acetonitrile at 30°C at a flow rate of 1 ml/min (fig.1A). Peptides were detected by measurement of absorption at 214 nm. The yields of peptides 1 and 2 (fig.1A) as determined by HPLC were 540 and 590 μg, respectively.

2.3. *Sequence analysis*

Edman degradation of peptides 1 and 2 was performed with an Applied Biosystems model 470A gas-phase sequencer [9] with some modifications. A fourth solvent (S1 = *n*-heptane) was included for washing of the filter for 0.5 min after coupling with phenylisothiocyanate. The conversion was carried out with 25% (v/v) trifluoroacetic acid. The phenylthiohydantoin amino acids (PTH-aa) were dissolved in 0.25 ml of methanol/acetonitrile (50:50, v/v) and dried in a Savant vacuum centrifuge for 10 min at 45°C. Dried PTH-aa were redissolved in 25 μl of acetonitrile containing methylthiohydantoin-tryptophan as internal standard. The PTH-aa were identified and quantified by reverse-phase HPLC on an Applied Biosystems PTH C-18 microbore column (2.1 × 220 mm) as described by the manufacturer. The column was mounted in a Hewlett-Packard liquid chromatograph model 1090 equipped with a programmable filter-photometer for detection at 263 and 314 nm (dehydro-derivatives of Ser and Thr). The detection limit of PTH-aa was 0.5 pmol.

2.4. *Peptide mapping*

Samples of yeast peptides 1 and 2 corresponding to 16 μg were dissolved in 200 μl of 50 mM Tris-HCl buffer (pH 7.4) containing 25 mM of Na_4EDTA. A solution of 10 μl of subtilisin A (Novo Industri A/S) containing 0.5 μg/μl in 50 mM Tris-HCl buffer (pH 7.4) was added. This corresponds to an enzyme to substrate ratio of 1:16, mol/mol. The digestions were carried out at 37°C for 18 h. Standards of human insulin and des(B-30)human insulin (Novo Industri A/S) were digested under the same conditions. The peptide mapping was carried out by the injection of 10 μl of the above digest mixtures onto a Macherey-Nagel 5 μm Nucleosil RP C-18 column (4 × 200 mm). The A and B buffers were 5% acetonitrile in 1.5 mM sulphuric acid and 45% acetonitrile in 1.5 mM sulphuric acid containing 0.15 M ammonium sulphate. The column was eluted with 5% B for 4 min and thereafter with a linear gradient to 45% B over 20 min at a flow rate of 1 ml/min at 30°C. Peptides were detected by measuring the absorption at 214 nm (fig.2).

2.5. *Assay for insulin*

Yeast peptides 1 and 2 were analyzed in a free fat

cell bioassay for insulin as described by Moody et al. [10] with the exception that the fat cells were taken from mice instead of from rats. In this type of assay, des(B-30)insulin has a potency of approx. 100% whereas otherwise modified insulins have a significantly reduced potency [11]. The potency of, e.g., des(A-21)insulin and desoctapeptide-(B23-30)insulin is less than 5% [11].

3. RESULTS

3.1. *Purification of secreted peptides*

The scheme used in the present study to purify insulin-like peptides from the fermentation broth consisted of three steps. The initial step was carried out mainly to concentrate the peptide material in the yeast culture supernatant. The subsequent immunoaffinity step on the anti-insulin Sepharose column retained only insulin-like peptides, which were finally purified in the HPLC step. Insulin-like molecules with e.g. incorrect disulphide bridges, or various insulin degradation products would not be absorbed on the immunoaffinity column. By the addition of ^{125}I-insulin (Novo Industri A/S) to the yeast supernatant, the yields in the different steps of the purification were found to be: 89% (LiChroprep RP-18 column), 95% (anti-insulin Sepharose column) and 70% (HPLC) corresponding to an overall yield of 59%. The eluate from the anti-insulin Sepharose column was found to contain two major insulin-like peptides (peptides 1 and 2, fig.1A), and three minor components. Only the peptides corresponding to the two major peaks were further characterized.

By HPLC analysis peptide 1 was found to coelute with a standard of human insulin (fig.1B) and peptide 2 was found to coelute with a standard of des(B-30)human insulin (fig.1C). Fig.1D shows the HPLC chromatogram obtained by the analysis of a mixture of the human insulin and des(B-30)human insulin standards.

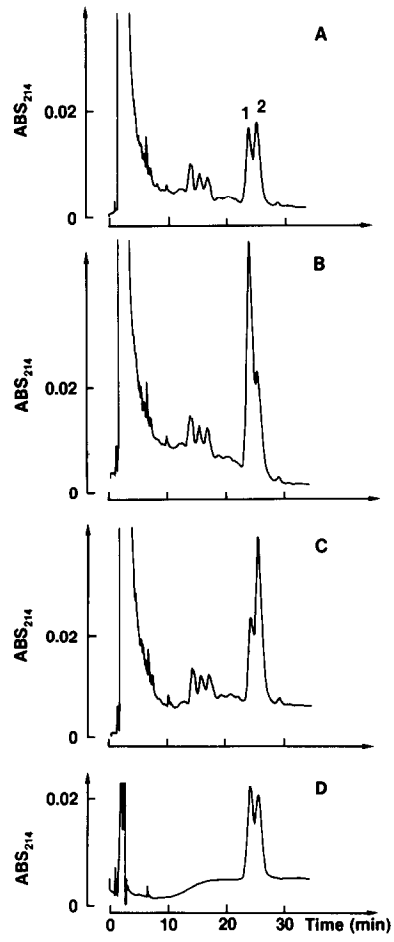

Fig.1. HPLC traces showing the coelution of yeast peptide 1 with human insulin and yeast peptide 2 with des(B-30)human insulin. Panel A, anti-insulin Sepharose eluate. Peptides 1 and 2 elute at the retention times of 24.01 and 25.38 min, respectively. Panel B, anti-insulin Sepharose eluate after the addition of 1 μg of human insulin. Panel C, anti-insulin Sepharose elutes after the addition of 1 μg of des(B-30)human insulin. Panel D, a standard mixture of 1 μg of human insulin and 1 μg of des(B-30)human insulin. All chromatograms were obtained under isocratic conditions (29.4% of acetonitrile).

3.2. *Structure analysis*

The results from the automated Edman degradation of yeast peptides 1 and 2 are shown in table 1. Both peptides were found to be two chain molecules. The amino acid sequences of peptides 1 and 2 were found to be identical to those of human insulin and des(B-30)human insulin, respectively.

In addition, peptide mapping of yeast peptides 1 and 2 was performed after digestion with subtilisin A, and the peptide maps were compared with those obtained from standards of human insulin and des(B-30)human insulin under the same conditions (fig.2). These analyses establish that the disulphide bond configurations in the two yeast peptides are identical to those of insulin.

Table 1

Automated Edman degradation of peptides 1 and 2[a]

Cycle no.	Peptide 1				Peptide 2			
	PTH-aa	Yield (pmol)	PTH-aa	Yield (pmol)	PTH-aa	Yield (pmol)	PTH-aa	Yield (pmol)
1	Phe	1688	Gly	1314	Phe	156	Gly	165
2	Val	1302	Ile	1218	Val	152	Ile	187
3	Asn	1023	Val	1338	Asn	318	Val	196
4	Gln	783	Glu	1381	Gln	144	Glu	238
5	His	483	Gln	957	His	61	Gln	162
6	Leu	1068	(Cys)[c]	–	Leu	103	(Cys)[c]	–
7	(Cys)[c]	–	(Cys)[c]	–	(Cys)[c]	–	(Cys)[c]	–
8	Gly	672	Thr	122	Gly	94	Thr	15
9	Ser	(105)[b]	Ser	(105)[b]	Ser	(22)[b]	Ser	(22)[b]
10	His	401	Ile	373	His	48	Ile	32
11	Leu	577	(Cys)[c]	–	Leu	66	(Cys)[c]	–
12	Val	537	Ser	75	Val	52	Ser	13
13	Glu	696	Leu	278	Glu	59	Leu	31
14	Ala	492	Tyr	232	Ala	57	Tyr	22
15	Leu	592	Gln	176	Leu	80	Gln	21
16	Tyr	509	Leu	329	Tyr	46	Leu	46
17	Leu	629	Glu	201	Leu	82	Glu	27
18	Val	413	Asn	162	Val	51	Asn	trace
19	(Cys)[c]	–	Tyr	174	(Cys)[c]	–	Tyr	11
20	Gly	284	(Cys)[c]	–	Gly	43	(Cys)[c]	–
21	Glu	380	Asn	101	Glu	39	Asn	trace
22	Arg	199			Arg	26		
23	Gly	179			Gly	35		
24	Phe	119			Phe	25		
25	Phe	154			Phe	31		
26	Tyr	133			Tyr	15		
27	Thr	23			Thr	9		
28	Pro	39			Pro	9		
29	Lys	33			Lys	6		
30	Thr	15						

[a] Average repetitive yield: 91.0% (peptide A), 90.5% (peptide B)
[b] Ser is present in both chains
[c] Cys is not determined by sequence analysis

From the results obtained by peptide mapping (fig.2), by sequence analysis (table 1) and by HPLC analysis (fig.1), it is concluded that yeast peptide 1 is identical to human insulin and yeast peptide 2 is identical to des(B-30)human insulin.

3.3. *Biological activity*

The biological potency of yeast peptides 1 as determined in the free fat cell assay was 97% ($P < 0.05$, confidence limit 93–102%) as compared to a human insulin standard (defined as 100%). When compared to a des(B-30)human insulin standard, yeast peptide 2 had a potency of 101% ($P < 0.05$, confidence limit 96–106%). These results are in agreement with the potency obtained by use of the corresponding pancreatic peptides [11].

4. DISCUSSION

In the present study the gene encoding the mini-

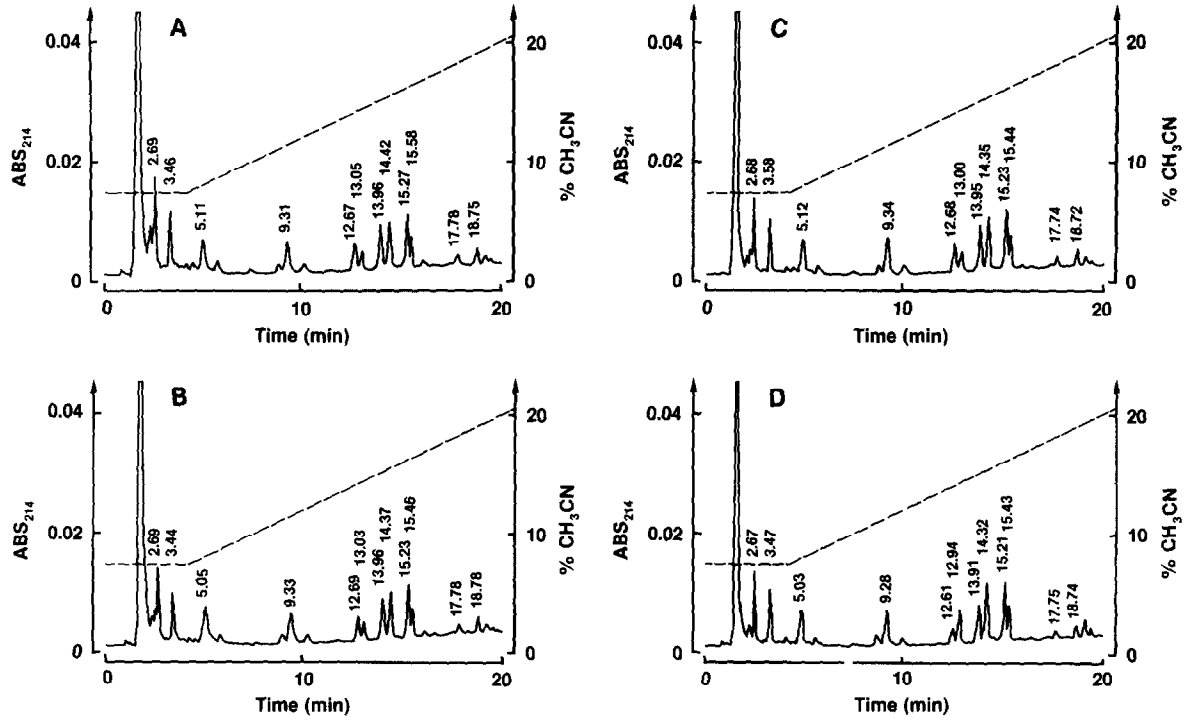

Fig.2. Peptide maps after digestion of peptides with subtilisin. Panels: A, yeast peptide 1; B, human insulin; C, yeast peptide 2; D, des(B-30)human insulin.

proinsulin molecule: B-Lys-Arg-Leu-Gln-Arg-Arg-A was transformed into *S. cerevisiae*. Two peptides were isolated in approximately equal amounts from the culture supernatant of the transformed yeast strain. By structural analysis as well as by biological activity the peptides were identified as human insulin and des(B-30)human insulin. We have previously detected only minor amounts of disulphide bridge-linked molecules secreted from yeast cells in which the intact proinsulin encoding gene had been inserted [6]. Thus, correct in vivo folding and disulphide bond formation seem to occur more easily in the mini-proinsulin molecule than in the intact proinsulin molecule. Furthermore, the rate of folding and disulphide bond formation in the mini-proinsulin molecule is sufficiently high to prevent extensive premature processing and consequent separation of the B-chain from the A-chain. Several insulin precursors containing a short peptide link between amino acids B-30 and A-1 were recently expressed and secreted from transformed yeast cells [6,12]. These types of molecules, e.g. B(1–29)-Ser-Lys-A(1–21) [12] and B(1–30)-Lys-Arg-A(1–21) [6], can be isolated as single-chained, disulphide bridge-linked molecules from the yeast culture supernatant. The single chain nature of these molecules is due either to the absence of processing sites between the B- and A-chains, or, when such sites are present, the single chain nature has been taken as evidence of the inaccessibility of the sites to the yeast processing enzyme system [6]. The present study shows that a short spacer peptide flanked by dibasic sequences placed between the B- and A-chains results in a precursor molecule which, in addition to its rapid folding, carries its proteolytic processing sites in a configuration sufficiently exposed to allow the in vivo conversion into two chain insulin. The processing in yeast of the Lys-Arg and Arg-Arg sequences of the mini-proinsulin molecule is most probably carried out by the Lys-Arg specific endopeptidase of *S. cerevisiae*, the KEX 2 gene product [13].

The generation of des(B-30)human insulin could be a result either of a trypsin-like cleavage after B-29 Lys or of a carboxypeptidase-like removal of

B-30 Thr. It has previously been shown that the Lys-Arg processing enzyme in yeast does not cleave after single basic amino acid residues [14], and a membrane-bound carboxypeptidase possibly involved in the removal of Lys and Arg during α-factor processing has been identified [14]. We find it therefore most likely that the membrane-bound carboxypeptidase is responsible for the removal of B-30 Thr. The reason for the enzyme not to remove B-29 Lys as well might be caused by the presence of Pro in position B-28.

ACKNOWLEDGEMENTS

We wish to express our appreciation to Per N. Jørgensen (Novo Biolabs) for providing monoclonal insulin antibodies. Ms A. Demandt, Ms D.M. Gundesen and Ms I. Hoegh are thanked for their technical assistance and Ms M. Petersen for the linguistic editing and the preparation of the manuscript.

REFERENCES

[1] Duntze, W., Stötzler, D., Bücking-Throm, E. and Kalbitzer, S. (1973) Eur. J. Biochem. 35, 357–365.
[2] Stötzler, D., Kiltz, H.H. and Duntze, W. (1976) Eur. J. Biochem. 69, 397–400.
[3] Kurjan, J. and Herskowitz, I. (1982) Cell 30, 933–943.
[4] Julius, D., Blair, L., Brake, A., Sprague, G. and Thorner, J. (1983) Cell 32, 839–852.
[5] Steiner, D.F., Docherty, K. and Carrol, R. (1984) J. Cell Biol. 24, 121–130.
[6] Thim, L., Hansen, M.T., Norris, K., Hoegh, I., Boel, E., Forstrom, J., Ammerer, G. and Fiil, N.P. (1986) Proc. Natl. Acad. Sci. USA 83, 6766–6770.
[7] Kawasaki, G. (1984) 12th International Conference on Yeast Genetics and Molecular Biology, Sept.17–24, 1984, Edinburgh, Scotland, abstr., P15.
[8] Sherman, F., Fink, G.R. and Hicks, J.B. (1981) Methods in Yeast Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[9] Hunkapiller, M.W., Hewick, R.M., Dreyer, W.J. and Hood, L.E. (1983) Methods Enzymol. 91, 399–413.
[10] Moody, A.J., Stan, M.A., Stan, M. and Gliemann, J. (1974) Horm. Metab. Res. 6, 12–16.
[11] Gliemann, J. and Gammeltoft, S. (1974) Diabetologia 10, 105–113.
[12] Markussen, J., Damgaard, U., Diers, I., Fiil, N., Hansen, M.T., Larsen, P., Norris, F., Norris, K., Schou, O., Snel, L., Thim, L. and Voigt, H.O. (1986) in: Peptides (Theodoropoulos, D. ed.) Walter de Gruyter, Berlin, in press.
[13] Julius, D., Brake, A., Blair, L., Kunisawa, R. and Thorner, J. (1984) Cell 37, 1075–1089.
[14] Achstetter, T. and Wolf, D.H. (1985) EMBO J. 4, 173–177.