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Production of human gastric lipase in the fission yeast *Schizosaccharomyces pombe*

(Recombinant DNA; homologous promoter; heterologous promoter; *adh1*; CaMV 35S; plasmid copy number; growth rate; lipolytic activity assay)

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SUMMARY

A cDNA encoding human gastric lipase (hGL) has been expressed on multicopy plasmids in the fission yeast *Schizosaccharomyces pombe* (*Sp*). Active lipase is secreted from transformants containing the h*GL* cDNA under the control of either the *Sp adh1* promoter (*Padh1*) or the plant cauliflower mosaic virus (CaMV) 35S promoter. Cell-wall-associated lipase activities are greatest in the early logarithmic growth phase and with *Padh1*. Western blot analysis indicates that a protein of identical molecular mass to natural hGL is secreted by *Sp*, although the major secreted product is of a higher molecular mass than either native hGL or recombinant hGL produced in the budding yeast *Saccharomyces cerevisiae* (*Sc*). Several distinct hGL are present within cells at all growth phases. Treatment of these proteins with endoglycosidase H gives rise to a single species equivalent in size to deglycosylated natural hGL, indicating that most of these are glycosylation intermediates. An hGL of similar molecular mass accumulates intracellularly in *Sp* when a modified version of cDNA is used which lacks the sequence encoding the natural secretory signal peptide. Production of hGL markedly slows the growth rate of *Sp*. The average copy number per cell of the plasmid expressing the h*GL* cDNA from the recombinant *Padh1* is 2–3, as compared with 11–12 for the control plasmid.

INTRODUCTION

Pancreatic lipase deficiency in humans can lead to the passage of undigested fat in the faeces (steatorrhoea) and malnutrition, and may occur in such conditions as cystic fibrosis, pancreatitis and alcoholism. It has been suggested that due to low intestinal pH levels, especially in cystic fibrosis sufferers, enzyme replacement therapy using an acid-stable lipase could improve fat digestion to a greater extent than presently used pancreatic lipase preparations (Abrams et al., 1984). Such an acid-stable lipase has been identified and purified from human gastric juice (Tiruppathi and Balasubramanian, 1982; Gargouri et al., 1986b). This has been shown to be of gastric origin, in contrast to the situation in rodents where preduodenal lipase is predominantly of lingual origin (DeNigris et al.,

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Abbreviations: Ab, antibody(ies); *adh1*, gene encoding alcohol dehydrogenase; Ap, ampicillin; bp, base pair(s); BSA, bovine serum albumin; cDNA, complementary DNA; CaMV, cauliflower mosaic virus; hGL, human gastric lipase; h*GL*, gene (DNA, RNA) encoding hGL; kb, kilobase(s) or 1000 bp; MCS, multiple cloning site(s); nt, nucleotide(s); *P*, promoter; PAGE, polyacrylamide-gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; ^{R}, resistant; re-, recombinant; rRNA, ribosomal RNA; *Sc*, *Saccharomyces cerevisiae*; SDS, sodium dodecyl sulfate; *Sp*, *Schizosaccharomyces pombe*; [], denotes plasmid-carrier state.

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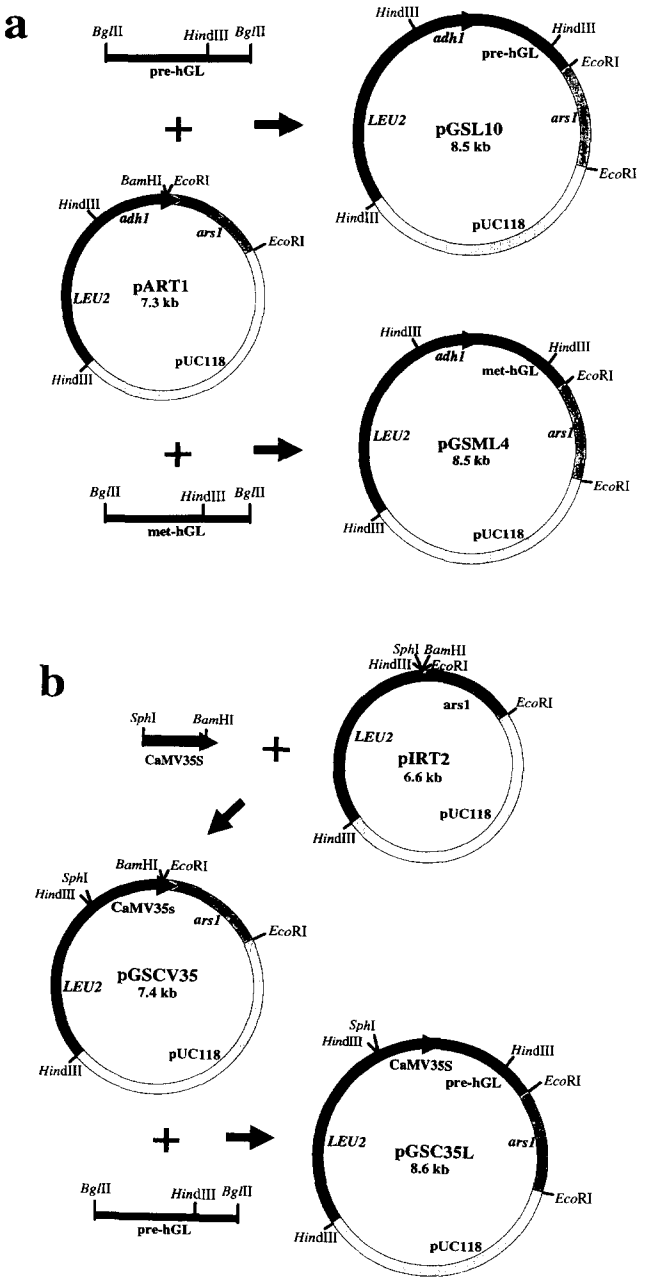

Fig. 1. Construction of hGL expression plasmids. (**a**) Construction of h*GL*-expression plasmids pGSL10 and pGSML4. Recombinant DNA methods were essentially according to Sambrook et al. (1989). The *Sp* expression vector pART1 which contains the homologous *Padh1* (McLeod et al., 1987) was digested to completion with *Bam*HI and treated with calf intestinal phosphatase. The cDNAs encoding hGL and met hGL originated from the vectors pMB1 and pYV3, respectively, and were isolated by complete *Bgl*II digestion (Bodmer et al., 1987). Purification of the cDNAs was carried out using 0.8% agarose gel electrophoresis and the fragments recovered using Geneclean™. The cDNAs were ligated into the pART1 expression vector to produce the plasmids pGSL10 (hGL) and pGSML4 (met hGL). (**b**) Construction of plasmid pGSC35L. The plasmid pBI121 (Clontech, Palo Alto, CA, USA) was digested to completion with *Sph*I + *Bam*HI. The CaMV 35S promoter fragment was purified by 0.8% agarose gel electrophoresis and recovered from the gel using Geneclean™. The *Sp* shuttle vector pIRT2 (based on pIRT1 (Booher and Beach, 1987) but with the *Sc LEU2* gene as a selectable marker) was similarly digested at the *Sph*I and *Bam*HI sites located within the MCS. The CaMV 35S promoter fragment was ligated into the digested pIRT2 to produce the expression vector pGSCV35. The *Bam*HI site downstream from the CaMV 35S promoter in plasmid pGSCV35 was then used for insertion of the h*GL* cDNA. The h*GL* cDNA was isolated by *Bgl*II digestion, 0.8% agarose gel purified as a 1.2-kb band and ligated into the *Bam*HI and phosphatase treated pGSCV35 expression vector to produce the plasmid pGSC35L.

1988). Therefore, hGL has potential for enzyme replacement therapy in cases of pancreatic lipase deficiency. Commercial production requires that hGL be produced of sufficiently authentic quality and in high quantity. To this end the cDNA of hGL has been cloned, by virtue of its homology with rat lingual lipase, and expressed in the budding yeast *Saccharomyces cerevisiae* (*Sc*) (Bodmer et al., 1987)

The fission yeast *Schizosaccharomyces pombe* (*Sp*) offers many of the advantages of the budding yeast *Sc* for molecular genetic manipulation, but evidence is accumulating that the cell and molecular biological features of the two yeasts differ greatly, and that, in many respects, *Sp* has greater similarities with higher eukaryotes than with *Sc* (Russell and Nurse, 1986). Similarities are exhibited between *Sp* and higher eukaryotes in *cis* and *trans* transcription factors (Russell, 1983; 1985; Jones et al., 1988; Swaminathan et al., 1993), intron splicing (Käufer et al., 1985), small nuclear RNAs (Brennwald et al., 1988; Kleinschmidt et al., 1990), centromere structure and function (Clarke, 1990; Polizzi and Clarke, 1991), and in the organisation of the cell cycle (Nurse, 1985). These features have increased interest in the investigation of *Sp* as a system for heterologous production of higher eukaryotic proteins. Recently, both human P-glycoprotein (Ueda et al., 1993) and the human D_{2S} dopamine receptor (Sander et al., 1994) have been successfully expressed using the fission yeast system.

In this paper we describe the investigation of *Sp* as a host for the production of hGL.

EXPERIMENTAL AND DISCUSSION

(a) Vectors and strains for the expression of h*GL* cDNA

The strong constitutive *Sp* promoter *Padh1* (Russell and Hall, 1983) was used to direct expression of the cDNA encoding pre-hGL. The cDNA was inserted, in the correct orientation downstream from *Padh1*, into the *Bam*HI site of the MCS of the *Sp* expression vector pART1 (McLeod et al., 1987) to produce the plasmid pGSL10 (see Fig. 1a for details). This plasmid also carries the *Sp ars1* element for stability, the *S. cerevisiae LEU2* gene, which is known to complement the *leu1-32* allele of *Sp* (Beach and Nurse, 1981) and the Ap^R gene for selection in *E. coli*. To evaluate intracellular production

of hGL in *Sp* a modified cDNA of hGL (met hGL) which lacks the secretion sequence (Bodmer et al., 1987) was inserted into the *Bam*HI site of pART1 to produce the plasmid pGSML4 (Fig. 1a).

The h*GL* was also expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter which also acts as a strong constitutive promoter in *Sp* (Gmünder and Kohli, 1989). For this construction the CaMV 35S promoter was first inserted into the MCS of the *Sp* vector, pIRT2 (Fig. 1b). The resultant plasmid pGSCV35 was used for the expression of h*GL* by inserting the h*GL* cDNA immediately downstream from the promoter to produce the plasmid pGSC35L.

Each of the above vectors was transformed into the *Sp leu1-32 h*$^-$ strain by the method of protoplasting (Beach and Nurse, 1981) and transformants selected on minimal medium.

(b) Analysis of hGL activity

Growth of transformed *Sp* on plates containing an emulsion of 0.75% (v/v) tributyrin (Sigma) was used as an initial test for the secretion of re-hGL. Secretion of active enzyme was seen as a zone of clearing surrounding the yeast colony after incubation at 30°C for 48 h (Fig. 2a). *Sp* cells transformed with pGSL10 (*Padh1*) and pGSC35L (CaMV 35S promoter) showed obvious zones of clearing, the diameter of the zone being greater with the *Padh1* construct (pGSL10). Cells transformed with pGSCV35 and pART1 showed very limited zones of clearing, equivalent to those by untransformed cells.

Lipase activity was determined potentiometrically (Gargouri et al., 1986a) using a tributyrin substrate under standard incubation conditions of pH 5.4 and 37°C. Due to the extreme susceptibility of hGL to irreversible interfacial denaturation at air/liquid interfaces in the absence of amphiphiles (Gargouri et al., 1986b), lipase activity was undetectable in the culture medium. Activity was also greatly reduced in cells broken by vigorous physical means. However, relatively high activity could be detected using whole-cell suspensions in the assay. Results from these assays are shown in Fig. 2b, which confirm that lipase activity is correlated with the presence of h*GL* cDNA. Activity levels are greatest in early exponential phase, decreasing by about one half at the onset of stationary phase and becoming negligible after 12 h in stationary phase. The data also confirm that the pGSL10 (*Padh1*) construct produces higher activity levels than the pGSC35L (CaMV 35S promoter) construct throughout each growth phase.

(c) Western blot analysis of re-hGL

Qualitative analysis of the re-hGL was carried out using Western blotting techniques. The transformed and

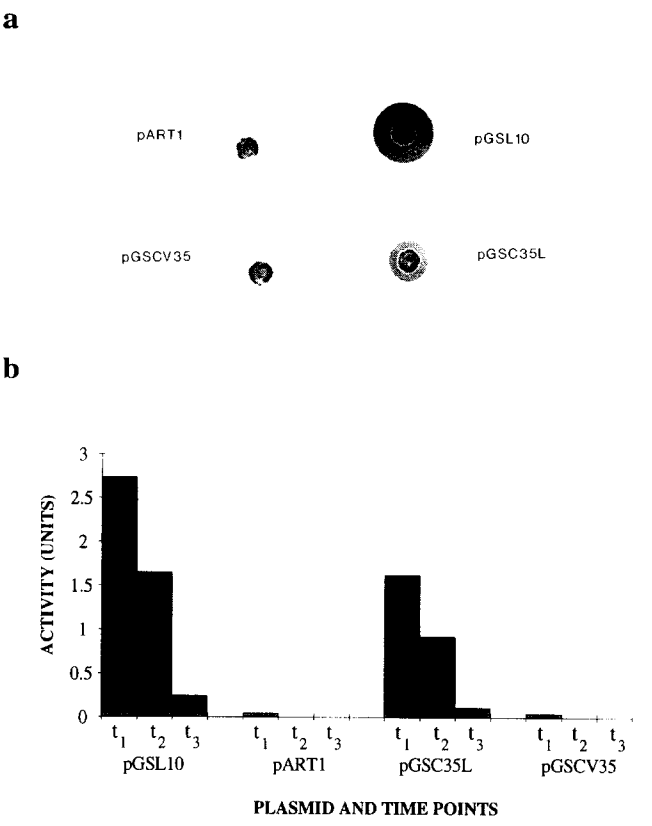

Fig. 2. re-hGL activity. (**a**) Detection of hGL activity using plate assays. *Sp* transformants containing the vectors pART1, pGSCV35, pGSL10 or pGSC35L were patched onto *Sc* minimal medium agar plates containing 0.75% tributyrin/0.1% Triton X-100/0.1% Tween-20. Cells were grown for 48 h at 30°C and the plate photographed using dark-field illumination. Zones of clearing indicate secreted lipase activity. (**b**) hGL activity associated with the cell wall of four *Sp* transformants (pGSL10, pART1, pGSC35L and pGSCV35) sampled at three different growth phases. The activity of whole cell suspensions in distilled water was measured potentiometrically using a pH-stat apparatus (Radiometer TTT-80). Cells were grown in *Sc* minimal medium and harvested by centrifugation at early logarithmic phase ($A_{600}=0.5$; t_1), late logarithmic phase ($A_{600}=2$; t_2) or after 12 h in stationary phase ($A_{600}=2.5$; t_3). Activity measurements are given as units/100 µl cells $A_{600}=50$.

control strains were grown under auxotrophic selection in shake-flask culture and samples collected at three growth phases. Material from lysed cells and culture supernatants was analysed by SDS-PAGE and Western blotting. Fig. 3 shows that the polyclonal hGL Ab recognises a number of protein species from each lipase-producing strain at all growth phases. There is no apparent difference in the quality of the protein produced using either promoter system, and no Ab-cross-reacting proteins are detected in the extracts or supernatants taken from cells transformed with either control plasmid. The two most prevalent species detected in culture supernatants are those of the highest molecular mass and together these form the predominant secreted species. The smaller of these is equivalent in size (50 kDa) to natural glycosylated hGL (Fig. 4, lane 9); therefore, the larger

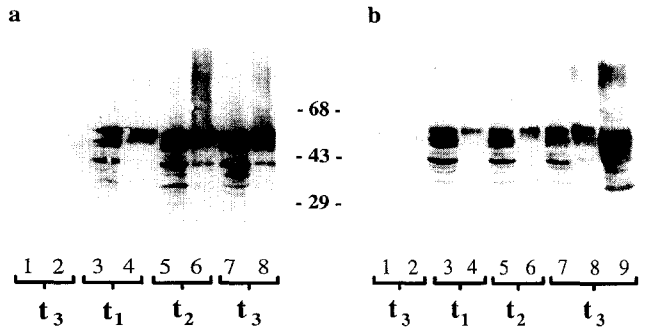

Fig. 3. Western blot analysis of hGL produced in *Sp* under the control of either the *Sp adh1* promoter (panel **a**) or the CaMV 35S promoter (panel **b**). Lanes 1, 3, 5, 7 and 9, cell extracts; lanes 2, 4, 6 and 8, concentrated culture supernatants. Extracts were taken from *Sp* cells transformed with: Panel **a**: lanes 1, 2, pART1; lanes 3–8, pGSL10. Panel **b**: lanes 1, 2, pGSCV35; lanes 3–8, pGSC35L; lane 9, *Sc*[pYC261]. The positions of markers (kDa) are shown. Cells from the experiment described in the legend to Fig. 2b were harvested by centrifugation at the times indicated and the cell pellets washed with water and resuspended in 1/50 original culture volume/10 ml Tris·Cl pH 7.5/1.2 M sorbitol. Cells were lysed by incubation with 1 mg Zymolyase per ml/2% (v/v) 2-mercaptoethanol/2 mM EDTA/1 mM PMSF at 30°C for 1 h and samples were subjected to electrophoresis. Culture supernatants (2.5-ml aliquots containing 1 mM PMSF) were desalted using PD-10 columns (Pharmacia) and freeze dried overnight. Samples were resuspended in Laemmli buffer (Laemmli, 1970) prior to electrophoresis. After 0.1% SDS-3–25% gradient PAGE, proteins were transferred electrophoretically onto nylon membrane (Hybond-N, Amersham) at 100 mA overnight. The membrane was probed with a rabbit polyclonal Ab raised against hGL and visualised using a peroxidase conjugated second Ab in conjunction with the ECL Western blotting detection system (Amersham).

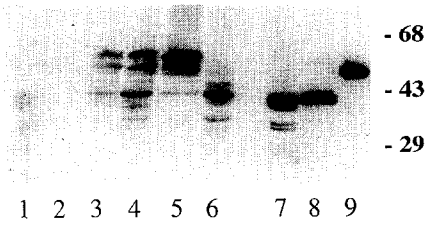

Fig. 4. Western blot analysis of hGL produced in *Sp*. Cells were grown, harvested and lysed as described in the legend to Fig. 3. All samples were taken from stationary phase cultures (t_3). Samples were analysed as described in the legend to Fig. 3. Lanes 1, 3, 4, 6 and 7, cell extracts; lanes 2, 5 and 8, concentrated culture supernatants; lane 9, natural hGL. Extracts were taken from *Sp* cells transformed with the following plasmids: lanes 1, 2, pART1 (control); lanes 3, 4, 5, pGSL10; lane 6, pGSL10 deglycosylated with endoglycosidase H (Boehringer-Mannheim); lanes 7, 8, pGSML4 (met hGL). Deglycosylation was carried out on boiled cell extracts using 10 munits endoglycosidase H in the presence of 0.005% SDS/1 mM PMSF/0.1% BSA/1 mM EDTA/50 mM Na·acetate buffer pH 5.5. The digestion mixture was incubated at 37°C overnight prior to electrophoresis.

protein is likely to represent an hGL product which is overglycosylated in *Sp*. In addition to these predominant species, a number of distinct smaller proteins are detected in the intracellular *Sp* samples (notably a 40-kDa species) which probably represent a mixture of glycosylation intermediates and degradation products. It is evident from lane 9 of Fig. 3 that the re-hGL proteins produced in *Sc* are within a similar size range but are more heterogeneous in size than those produced in *Sp*.

The demonstration that *Sp* produces and secretes hGL of identical size to that of the natural product, and that hGL produced in fission yeast is more homogeneous than that produced in *Sc*, indicates that *Sp* should be seriously considered as a host system for the production of glycosylated proteins from higher eukaryotes.

(d) Western blot analysis of both deglycosylated re-hGL and met hGL

To assess whether the multiple-molecular-mass forms of hGL produced in *Sp* were due to different degrees of glycosylation a sample of cell extract containing hGL produced from pGSL10 was treated with endoglycosidase H to remove *N*-linked glycosyl residues (Fig. 4). This treatment results in a major protein species of approx. 40 kDa, similar in molecular mass to deglycosylated natural hGL (Bodmer et al., 1987). This confirms that the two high-molecular-mass hGL proteins produced in *Sp* (lanes 3–5) are glycosylated forms. The low-level proteins intermediate in size between these and the 40-kDa deglycosylated species are therefore likely to be glycosylation intermediates, and the species of <40 kDa must represent aberrantly synthesized lipase protein or degradation products. Lane 7 shows the hGL protein produced in *Sp*[pGSML4], i.e., containing cDNA encoding met hGL. The absence of a signal sequence on this protein should prevent the normal secretion and glycosylation pathway being followed. As expected, a single major protein is detected of similar size to the deglycosylated protein in lane 6, confirming the conclusions above about the nature of hGL glycosylation. The slightly smaller size of met hGL could be due to proteolytic clipping of this protein. The presence of met hGL in the culture supernatant is probably due to lysis of some of the very slow-growing pGSML4 transformants.

(e) Effect of hGL production on cell growth

Cells transformed with the control plasmid pART1 or with the hGL expression plasmid pGSL10 were grown in minimal medium in shake-flask cultures at 30°C with continuous subculturing to maintain the cells in logarithmic phase. Mean doubling times of 3 h 35 min (pART1) and 5 h 48 min (pGSL10) indicated a significant reduction in growth rate for the hGL-producing strain. A similar reduction in growth rate is observed during hGL cDNA expression in *Sc* (N. Weir, J. Bell, T. Crabbe and E.F.W., unpublished results).

(f) Effect of hGL production on plasmid copy number

During continuous subculturing of the strains containing the plasmids pGSL10 and pART1 samples were taken and total DNA extracted from the cells. DNA was subjected to restriction endonuclease digestion and Southern blotting using the *Padh1* as a probe (data not shown). The resulting autoradiograph was analysed by densitometric methods, with the single chromosomal copy of *adh1* being used as an internal standard. Results indicated that the mean level of pART1 remained at a constant 11–12 copies per cell, whereas that of pGSL10 was significantly lower, at less than 3 copies per cell. These results indicate that there is a strong selection against high level hGL production in *Sp*. This is consistent with the dramatically increased doubling times of cultures producing hGL when compared to non-producers.

(g) Conclusions

(*1*) Plasmids were constructed for the expression of h*GL* cDNA under the control of a homologous promoter and a heterologous promoter in *Sp*.

(*2*) hGL was produced and secreted in an active form under the control of both promoters. The level of expression was higher using the *adh1* promoter compared to expression with the CaMV 35S promoter.

(*3*) *Sp* produces and secretes a recombinant hGL of identical molecular mass to natural hGL, although the major secreted form is a higher apparent molecular mass. hGL produced in *Sp* is more homogeneous than that produced in *Sc*.

(*4*) Treatment of the recombinant protein with endoglycosidase H indicates that it is glycosylated by *Sp*, and the deglycosylated form is of a similar molecular mass to that of met lipase expressed in the same system.

(*5*) The production of hGL has a detrimental effect on growth rates, causing a >60% increase in doubling time for a culture in logarithmic phase.

(*6*) The presence of the h*GL* cDNA in the expression plasmid results in a >75% reduction in plasmid copy number when compared to the control plasmid.

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