Heterologous Protein Secretion from Yeast

Robert A. Smith, Margaret J. Duncan, Donald T. Moir

Summary. Secretion of calf prochymosin from yeast yields fully activable zymogen while production in the yeast cytoplasm yields insoluble, unactivable enzyme with aberrant disulfide bonding. Factors that increase the efficiency of secretion of prochymosin from yeast are use of a yeast secretion signal sequence, integration of the transcriptional unit into the yeast genome, and specific mutations in a number of host cell genes. In combination, these factors increase the secretion of calf prochymosin from less than 1 percent to more than 80 percent of the amount produced. Host mutations that increase prochymosin secretion also increase bovine growth hormone secretion more than 15-fold. These discoveries may be generally useful for production of many secreted mammalian proteins made inside microorganisms as insoluble aggregates.

The yeast *Saccharomyces cerevisiae* has recently gained popularity as a host for heterologous gene expression and protein secretion. This interest is due both to the ease and favorable economics of yeast fermentation, developed over years of industrial experience, and to the rapid progress made in the molecular genetics of the organism. The ability to introduce exogenous DNA into the yeast genome, coupled with advancements in molecular cloning techniques and understanding of the genetics and physiology of protein secretion from yeast cells (*1*), has led to the development of strains that produce and secrete such proteins as α-interferon (*2*), epidermal growth factor (*3*), and β-endorphin (*4*).

In this article, we describe the development of yeast strains that secrete calf prochymosin, the inactive precursor of chymosin (also known as rennin), which is used in the manufacture of cheese. Obvious advantages to be gained from the secretion of prochymosin, as opposed to its production in the cytoplasm of microorganisms, include the ease of continuous fermentation and processing and the relatively high initial purity of the desired protein product. However, we are interested in the secretion of prochymosin primarily because secreted prochymosin is fully activable to chymosin, unlike cytoplasmically produced prochymosin, which is largely insoluble and unactivable (*5*). Other naturally secreted mammalian proteins, such as bovine growth hormone (*6*), human tissue plasminogen activator (*7*), and human γ-interferon (*8*), are also insoluble and inactive when produced in the cytoplasm of microorganisms. Secretion, therefore, should play a major role in future industrial fermentation processes.

A secreted protein follows a specific pathway in the cell. Transit through this pathway may or may not occur as efficiently for heterologous proteins as for homologous ones. In this article, we discuss some of the parameters affecting the efficiency of secretion from yeast. Using calf prochymosin as a model for yeast secretion of heterologous proteins, we present results on the effect of promoter strength, gene dosage (both as plasmid copies and as integrated copies), secretion signal, and host mutations on the amount of protein production and secretion. The results indicate that heterologous protein secretion from yeast becomes saturated at a surprisingly low level. However, we show that the saturation level can be increased significantly by changes in the secretion signal and the mode of maintenance of the transcriptional unit, as well as by mutations in the host genome.

Robert A. Smith, Margaret J. Duncan, and Donald T. Moir are members of the Department of Molecular Genetics, Collaborative Research, Inc., Lexington, Massachusetts 02173.

Secreted Prochymosin Fully Activable to Chymosin

Prochymosin has been a prime target for production in microorganisms because of its industrial utility and limited availability. Indeed, strains of both yeast and *Escherichia coli* that produce calf prochymosin cytoplasmically have been described (*9, 10*). A striking characteristic of the prochymosin produced by all of these strains is that it is largely insoluble and unactivable. It can be isolated only after solubilization with protein denaturants. Even after removal of the denaturant and refolding of the protein, incubation of this prochymosin at low *p*H converts less than 25 percent to active chymosin (*11*). Native calf prochymosin is autocatalytically activated to chymosin at low *p*H with nearly 100 percent efficiency by removal of 42 amino acid residues from the amino terminus (*12*).

The structure of insoluble prochymosin remains obscure because it can be examined only after denaturation. Nevertheless, it is clear that one or more of the three disulfide bonds present in the native molecule do not form, or form incorrectly, in the insoluble product found in yeast and *E. coli*. In polyacrylamide gels containing sodium dodecyl sulfate, the electrophoretic mobilities of prochymosin derived from calves and prochymosin produced in yeast cells are identical after reduction with dithiothreitol. Without prior reduction, however, the yeast-produced material migrates more slowly than the calf prochymosin. The slower migration is consistent with a more extended structure for the protein made in yeast, which may result because conditions inside cells are too reducing to permit disulfide bond formation. The fact that most proteins containing disulfide bonds are extracellular suggests that the secretion pathway may favor disulfide bond formation. The inactivity of intracellular prochymosin from yeast and *E. coli* is not surprising because the protein is normally made with a 16-residue amino-terminal secretion signal (*13*) and is secreted into the fourth stomach of the calf.

We achieved secretion of prochymosin from yeast cells by use of the secretion signal from yeast invertase, and we found that the solubility and activability of prochymosin are then identical to those of native calf prochymosin (*5*). A segment of DNA containing the *SUC2* (invertase) transcriptional promoter region, the coding region for the amino-terminal secretion signal, and codons for the first 11 amino acid residues of mature invertase were fused in translational reading frame with a prochymosin-encoding DNA fragment and incorporated

into a plasmid vector (Fig. 1A) (*5, 14*). In yeast the expression product from this construct is an invertase-prochymosin fusion protein that after removal of the signal peptide during secretion is processed at low *p*H in the same way that natural calf zymogen is processed to yield active chymosin (Fig. 1B and lane g in Fig. 2).

Prochymosin production by yeast strains containing this plasmid, and by others in which the *SUC*2 promoter and secretion signal have been replaced by different promoters or secretion signals (Fig. 1A), is shown in Table 1. A comparison of strains with similar total prochymosin expression levels (experiments 1 and 4, 2 and 5, and 3 and 6 in Table 1; activable enzyme levels from wild-type cells) shows that the addition of the invertase secretion signal results in the production of secreted activable prochymosin, in addition to an amount of internal activable prochymosin similar to that produced from constructs without the secretion signal. Most of the antigen produced, either with or without a secretion signal, remains internal and cannot be activated efficiently to chymosin (experiments 1 to 6 in Table 1; internal activable enzyme levels in wild-type cells). All of the secreted prochymosin, however, can be activated to chymosin (see below). Other secretion signals, both from yeast and from the native gene, are less effective than the invertase signal (experiments 7 to 9 in Table 1; secreted levels from wild-type cells).

Processing and Glycosylation of Secreted Prochymosin

Processing of the invertase-prochymosin fusion protein during its production and secretion is illustrated schematically in Fig. 1B and by gel electrophoretic analysis in Fig. 2. In wild-type cells, most of the fusion protein is internal. This fusion protein (lane a in Fig. 2) is glycosylated because cell extracts treated with endoglycosidase H (lane b in Fig. 2) contain a smaller protein, as do extracts from cells treated with tunicamycin (lane c in Fig. 2). Incubations with endoglycosidase H for varying lengths of time indicate that only one glycosylation site is used even though there are three potential sites for asparagine-linked glycosylation in this fusion protein—one in the invertase portion of the molecule and two in the chymosin portion [amino acid residues 310 and 349 (*13*)]. Activation of the invertase-prochymosin fusion protein to chymosin at low *p*H removes all of the carbohydrate, demonstrating that the sugar moieties must be present on the glycosylation site in the invertase portion of the fusion protein (lane g in Fig. 2). It is likely that certain asparagines within Asn-x-Thr/Ser sequences of a protein are not glycosylated because they are inacessible to the glycosylation enzymes (*15*). The fact that identical sequences in chymosin fail to be glycosylated in both the calf and yeast cells suggests that folding of the protein chain during secretion is similar in the two organisms.

The fusion protein, which does not contain carbohydrate because of treatment with endoglycosidase H or production in cells treated with tunicamycin, is only slightly larger than native calf prochymosin (lane d in Fig. 2). Compared to the much greater difference in mobility between prochymosin and chymosin (lanes d and h in Fig. 2), reflecting a difference of 42 amino acid residues, the slight mobility difference between deglycosylated invertase-prochymosin and native calf prochymosin (lanes c and d in Fig. 2) is consistent with the loss of the 19 amino acid residues of the invertase secretion signal and the retention of the 11 amino acid residues of mature invertase. Because the entire invertase secretion signal and mature invertase junction region are present on the initial product, it is likely that processing of the secretion signal has occurred at the normal junction. However, we have not determined the precise processing point of the secretion signal.

The primary secreted product appears to be heavily glycosylated, but is not visible in lane e of Fig. 2. When an equivalent amount of secreted material is first treated with endoglycosidase H, an intense band is visible in the region of the gel corresponding to the unglycosylated invertase-prochymosin fusion protein (lane f of Fig. 2). When more untreated secreted material is loaded on the gel, a broad region of antigenic activity can be seen in the high molecular weight portion of the gel. This heterogeneous electrophoretic mobility is analogous to that seen with secreted invertase (*16*) and is probably due to varying degrees of mannose outer chain addition. These additional mannose residues may interfere with antibody binding or electrophoretic transfer to nitrocellulose. Internal invertase-prochymosin appears to contain only core oligosaccharide because it migrates as a distinct band that is not altered in antigenic activity by endoglycosidase H treatment (lanes a and b in Fig. 2).

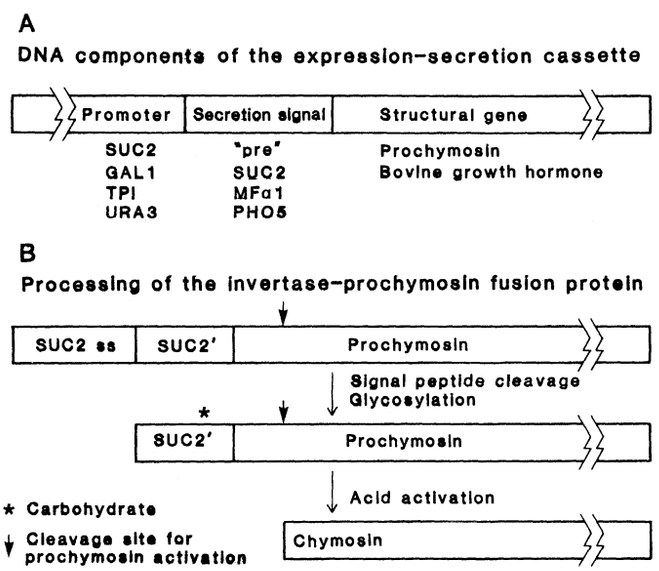

Fig. 1. (A) DNA components of the expression-secretion cassette. Several autonomously replicating yeast plasmids [see pCGS40 in (*9*)] were constructed in which three elements—the promoter, secretion-signal coding region, and structural gene—were varied. The *SUC*2 (*24*) and MFα1 (*25*) secretion signals also include regions of the structural genes preceding convenient restriction endonuclease access sites (Ava II and Hind III, respectively). The synthesis of the *PHO5* signal sequence encoding DNA was based on the published sequence (*26*). The designation "pre" refers to the DNA encoding the native secretion signal present on prochymosin or BGH complementary DNA. Access to the prochymosin encoding portion of the complementary DNA was obtained as previously described (*9*). The Met-Met-Ala-Ala initiating codons of pre-BGH were replaced with Met to yield a slightly truncated pre-BGH gene. The promoters are approximately 1-kb DNA segments found upstream from the following genes: *TPI* (*27*), with a 3′ end at −18 relative to the ATG translation initiation codon; *GAL*1, as previously described (*9*); *SUC*2, from plasmid pRB58 (*28*); *URA3*, from plasmid pRB71 (*29*), with a 3′ end at +12 relative to the ATG. (B) Processing of the invertase-prochymosin fusion protein. The initial translation product is a fusion of the *SUC*2 secretion signal and 11 amino acid residues of the invertase structural gene to prochymosin [see (*5*) for details]. At least four processing steps are observed: (i) the secretion-signal peptide is removed, (ii) a single core carbohydrate unit is added, (iii) the outer mannose chains are added, and (iv) the secreted fusion protein is activated to chymosin by a low *p*H incubation.

All of the activated secreted invertase-prochymosin exhibits an electrophoretic mobility identical to that of natural calf chymosin (lanes g and h in Fig. 2). No additional invertase-prochymosin is apparent after endoglycosidase H treatment, and no carbohydrate is present on the chymosin portion of the molecule. The amount of chymosin predicted from the milk-clotting activity [assuming a specific activity of 100 unit/mg; (*17*)] is consistent with the amount of secreted antigen detected by immunoblot. Therefore, all secreted prochymosin can be converted to active chymosin. This result is striking in comparison with the extremely poor activability of internal prochymosin, made both with and without a secretion signal sequence (experiments 3 and 6 in Table 1; internal levels from wild-type cells).

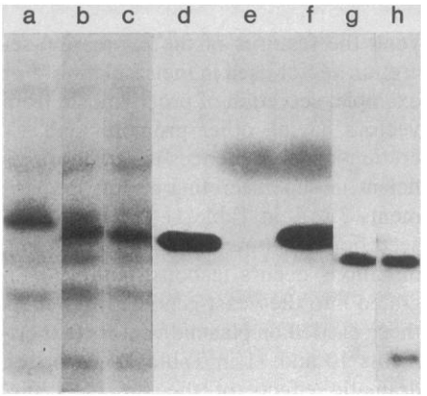

Fig. 2. Processing of invertase-prochymosin in the yeast secretion pathway. Internal invertase-prochymosin in crude extracts (100 μg of protein) or secreted invertase-prochymosin in culture broth (2 ml concentrated by ultrafiltration) from yeast cultures (2×10^7 cell/ml) was subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Chymosin-related protein species were detected by immunoblot (*30*), for which rabbit antiserum against purified calf chymosin was used. Control immunoblots performed on yeast cell extracts (300 μg protein per lane) containing no prochymosin revealed no significant cross-reactivity of the antiserum with yeast proteins in the chymosin or prochymosin region of the gel [lane B of figure 5 in (*9*)]. Internal invertase-prochymosin made from a *SUC2*-promoted construct (experiment 5 in Table 1) in cells derepressed by growth for 3 hours in YNB (*31*) containing 0.05 percent glucose and 3 percent glycerol is shown untreated (lane a) and treated (lane b) with endoglycosidase H (*16*). The protein produced under derepressed conditions in the presence of tunicamycin (20 μg/ml) is shown in lane c. Secreted invertase-prochymosin produced from a *TPI*-promoted construct (experiment 6 in Table 1) in cells grown in succinate-buffered YNB (*31*) is shown before (lane e) and after (lane f) treatment with endoglycosidase H. The secreted product (160 ng of protein) of a strain carrying the *SUC2*-promoted construct derepressed on YNB agar plates (*31*) is shown in lane g. Both YNB and succinate-buffered YNB liquid media contained bovine serum albumin (25 μg/ml) to stabilize secreted chymosin. The diffusely stained region above the prochymosin band in lanes e and f is bovine serum albumin, which, at this heavy loading, shows some cross-reactivity with the antiserum. Calf prochymosin (lane d, 34 ng) and calf chymosin (lane h, 25 ng) are shown as markers.

Limitations on the Efficiency of Prochymosin Secretion

These results demonstrate that secretion of prochymosin overcomes the insolubility and inactivity associated with the cytoplasmically produced protein. However, they also show that less than one-tenth of the invertase-prochymosin made by the cells under these conditions is secreted. Replacement of the *SUC2* promoter with the stronger promoter triose phosphate isomerase (*TPI*) increases the expression level to 0.25 percent of total cell protein, but does not significantly increase the amount secreted (experiments 5 and 6 in Table 1; levels secreted from wild-type cells).

Two observations indicate that there is a limitation in the secretion pathway itself. First, protein secreted into the medium contains both core oligosaccharide and the outer-chain carbohydrate added in the Golgi apparatus of the cell. By contrast, most of the intracellular fusion protein contains only the core oligosaccharide added in the endoplasmic reticulum. Because very little of the fully glycosylated protein accumulates inside, it would appear that it is transported rapidly to the outside of the cell. These observations suggest that translocation of core oligosaccharide–containing protein from the endoplasmic reticulum to the Golgi, transit of protein through the Golgi, or the addition of outer-chain carbohydrate in this organelle may be a rate-limiting step in the secretion of invertase-prochymosin.

Second, indirect immunofluorescence studies indicate that a substantial fraction of the core glycosylated invertase-prochymosin within the cell is located in the cell vacuole (*14*). A rate-limiting Golgi-associated step in the secretion pathway might cause excess protein to be diverted to the vacuole. Alternatively, proteins that are "foreign" to yeast may contain insufficient sequence information to ensure their efficient secretion; thus, the vacuole may be a repository for protein that cannot be sorted properly.

In summary, prochymosin-secreting yeast cells appear to direct all of the prochymosin they produce to the secretion pathway, but they secrete only a small fraction of it. In a well-characterized pathway, the rate-limiting step could be accelerated by overproduction of the enzyme catalyzing it. In this case, however, the secretion pathway is complex, our understanding preliminary, and the actual rate-limiting step ill-defined.

Supersecreting Mutants

To identify mutant yeast strains with elevated secretion levels, we chose to take a general mutagenesis approach coupled with a suitable screen for the desired phenotype. We recently succeeded in isolating a number of mutant yeast strains that are capable of secreting a larger proportion of the prochymosin they produce (*18*). We have called them "supersecreting" mutants, and where it is clear that the supersecreting phenotype is due to a single gene mutation, we have assigned the designation *ssc* (for supersecretion) to that mutation.

The isolation of supersecreting strains of yeast was greatly facilitated by the development of a rapid screening assay that allows estimation of the relative amount of chymosin secreted by individual yeast colonies. This assay relies upon the fact that a yeast colony which secretes prochymosin leaves a "footprint" of secreted material on the surface of the nutrient agar plate on which it grows. This secreted material, which is activated by the low *p*H of the medium, can be assayed by overlaying the surface of the plate with a mixture of milk and molten agarose after the yeast colonies have been removed. The speed with which opaque regions of clotted milk form, and their size and intensity, are related to the amount of chymosin secreted by individual colonies. Thus, colonies that secrete more prochymosin can be distinguished from colonies that secrete less.

This assay was used to identify 39 supersecreting strains of yeast from a collection of approximately 120,000 mutagenized colonies. All of the mutant strains produce the same total amount of prochymosin as the parent strain, but some secrete as much as eight or ten times the wild-type amount. [A genetic analysis of these strains will be described in more detail elsewhere (*19*).] Both partially dominant and recessive mutations were obtained. The recessive mutations have been analyzed extensively, and they behave as simple Mendelian traits through several backcrosses. Complementation analysis of the recessive mutations indicates that mutations in at least four genes can lead to a superse-

creting phenotype, but mutations in two genes in particular—designated *SSC*1 and *SSC*2—are the strongest and most easily manipulated alleles.

To determine the additivity of the supersecretion effects, we constructed haploid yeast strains that contain both *ssc*1 and *ssc*2 mutations. Such doubly mutant strains secrete prochymosin more efficiently than either *ssc*1 or *ssc*2 single mutants (experiment 6 in Table 1). Other strains with particularly high secretion efficiencies could also be constructed by combining supersecreting mutations from other complementation groups. These results are important because they imply that each of a number of mutational changes can have significant independent, additive effects on the intracellular distribution of a protein such as prochymosin.

Supersecreting Mutations Are Effective for Other Proteins and Signals

We have tested the general utility of supersecreting strains in a number of ways. Clearly, their effects extend beyond the features of the expression-secretion vector used in their isolation. For example, secretion of prochymosin from vectors having other promoter and secretion-signal sequences is also more efficient in supersecreting strains (experiments 7 to 9 in Table 1). The increased secretion resulting from supersecreting mutations occurs in constructions integrated into the yeast genome, as well as those carried on plasmid vectors (experiments 10 and 11 in Table 1). Also, the dramatic effects of the supersecreting mutations are not limited to laboratory shake-flask cultures; rather, the yield of secreted invertase-prochymosin is proportional to cell number up to commercially useful cell densities of a least 25 g/liter (dry weight), resulting in secretion into the growth medium of 20 mg per liter of activable prochymosin, or about 80 to 85 percent of the prochymosin produced by the cells (*20*). Finally, some mutant strains selected solely on the basis of increased prochymosin secretion also have an increased secretion capacity for at least one other foreign protein. We introduced an expression vector for the secreted precursor of bovine growth hormone (pre-BGH) into supersecreting strains and found that the *ssc*1-1 mutation increases the secreted yield at least tenfold, and *ssc*2-1 exhibits a detectable but smaller effect (experiment 12 in Table 1).

Chromosomal Integration Improves Secretion Efficiency

Perhaps one of the most surprising observations we have made is that the efficiency with which a yeast strain secretes prochymosin can be affected by the manner in which the prochymosin gene is maintained in the cell. In particular, when the gene for prochymosin fused to a yeast promoter and secretion signal sequence is integrated into a yeast chromosome, a considerably greater proportion of the prochymosin is secreted than when the same promoter–signal sequence–prochymosin construction is introduced on a multicopy plasmid vector. Under conditions in which plasmid constructions result in a secretion efficiency of 1 to 2 percent, integrants typically secrete 8 to 10 percent of the pro-

Table 1. Heterologous protein production and secretion. Extrachromosomal 2-μm-based plasmid constructions described in Fig. 1 were introduced into one or more of the following yeast host strains: CGY80 (*MAT*a *his*3 *ura*3-52 *leu*2-3,112 *trpl*-289), CGY150 (*MAT*α *leu*2-3 *ura*3-52), CGY339 (*MAT*α *his*4-29 *ura*3-52 *pep*4-3), CGY434 (*MAT*α *his*4-519 *ura*3-50 *pep*4-3), and supersecreting mutants derived from CGY339 by ethyl methane sulfonate mutagenesis and backcrossed to wild-type laboratory strains. Constructions on YIp5 vectors (*32*) were integrated at three different yeast loci by means of sequence homology present on the vector; multiple integrants were obtained by genetic crosses. Transformants were grown in YNB (*31*) with appropriate amino acid supplements to a density of about 2×10^7 cells per milliliter and harvested by centrifugation. Cells were lysed with glass beads, and extracts were prepared by treatment with trichloroacetic acid according to method B (*9*). The total antigen level (both inside and outside the cells) was measured by immunoblot (*30*) with appropriate dilutions of samples and standards (purified from the native source); the total antigen level varied with different promoters and signal sequences but was independent of the host strain in every case examined. Levels of total antigen and activable enzyme are expressed as milligrams or micrograms per gram of soluble yeast protein, which was determined by means of a commercial reagent (Bio-Rad). Chymosin milk-clotting activity was determined after activation of prochymosin by incubation in 40 m*M* lactic acid and neutralization with NaOH (*9*). Activity measurements were converted to micrograms of activable enzyme assuming a specific activity for chymosin of 100 units per milligram (*17*). Internal activity was measured after activation of cell extracts prepared in 50 m*M* tris-HCl [method A in (*9*)]. Secreted protein was isolated by growing cells on an agar plate containing YNB with appropriate amino acid supplements for 3 days at 30°C, removing cells, and washing them with 50 m*M* sodium phosphate, *p*H 5.8. The activity was measured in the wash-buffer supernatant. Acid activation was unnecessary because the poorly buffered YNB medium reaches sufficiently low *p*H to activate all prochymosin secreted. This assay is convenient but underestimates the level of secretion compared with that observed in liquid culture by a factor of about 2 because some product diffuses into the agar layer beneath the cells. Abbreviations: P, plasmid; I, integrant; 4×I, four-copy integrant; pC, prochymosin; BGH, bovine growth hormone; *SUC*2(33) indicates an in-frame addition (33 bp, in this case) of a yeast gene (*SUC*2, in this case) between the indicated secretion-signal coding region and prochymosin; *SSC*X denotes a partially dominant supersecreting mutant whose complementation group has not been established.

Exp.	Promoter	Secretion signal	Gene	Vector	Total antigen produced (mg/g)	Level of activable enzyme [μg/g (percent of antigen)]					
						Internal	Secreted				
						Wild type	Wild type	*ssc*1	*ssc*2	*ssc*1*ssc*2	*SSC*X
1	*GAL*1		pC	P	2.5	10 (0.4)					
2	*URA*3		*URA*3(12)pC	P	0.7	4 (0.5)					
3	*TPI*		pC	P	2.0	15 (0.8)					
4	*GAL*1	*SUC*2	*SUC*2(33)pC	P	3.0	18 (0.6)	27 (0.9)	65 (2.2)	141 (7.9)	238 (7.9)	177 (5.9)
5	*SUC*2	*SUC*2	*SUC*2(33)pC	P	0.5	<2 (0.5)	21 (4.2)	50 (10)		256 (51)	
6	*TPI*	*SUC*2	*SUC*2(33)pC	P	2.5	18 (0.7)	23 (0.9)	264 (10)	274 (11)	675 (27)	126 (5.0)
7	*GAL*1	pre	pC	P	2.5		<6 (0.2)	117 (4.7)	46 (1.8)	275 (11)	
8	*MF*α1	*MF*α1	*MF*α1(267)pC	P	2.0		11 (0.6)		68 (3.4)		43 (2.2)
9	*GAL*1	*PHO*5	pC	P	3.0		<6 (0.2)	268 (8.9)	129 (4.3)	390 (13)	45 (1.5)
10	*TPI*	*SUC*2	*SUC*2(33)pC	I	0.7		82 (12)	246 (35)	264 (38)		
11	*TPI*	*SUC*2	*SUC*2(33)pC	4×I	3.0		240 (8.0)	1382 (45)	1224 (40)		
12	*GAL*1	pre	BGH	P	3.0		3 (0.1)*	36 (1.2)*	4 (0.2)*	40 (1.6)*	

*Level of secreted antigen, with percentage of total antigen in parentheses, as determined by immunoblot (*30*).

chymosin they produce (experiments 6, 10, and 11, in Table 1; levels secreted from wild-type cells).

Multicopy plasmids might be expected to display a lower secretion efficiency because of their higher copy number and correspondingly higher gene expression level and larger reservoir of unsecretable prochymosin. However, this hypothesis is clearly not correct because we have constructed strains with several integrated copies of invertase-prochymosin fusion genes. Such strains produce approximately the same absolute amount of prochymosin as strains containing the same construction on a multicopy plasmid, but they secrete at least four times as much (experiments 6 and 11 in Table 1).

Thus, chromosomal integration appears to be the method of choice for introducing foreign genes into yeast—especially if secretion of the products is desired. Integration results in improved secretion efficiency for prochymosin and presumably also for other gene products. The intrinsic genetic stability of integrated constructions is well established (*21*). Unlike most plasmid constructions, these chromosomally integrated constructions do not require selective pressure for maintenance, thus removing restrictions on the type of growth medium that can be used and allowing growth of cells in continuous culture for long periods. Finally, the major anticipated shortcoming of the integrated constructions—namely, their relatively low copy number—does not diminish the usefulness of integration as a gene maintenance strategy because, in practice, many copies of the transcriptional unit are only marginally more productive than a few copies (*14*).

Discussion

We have shown that secretion of prochymosin is critical for obtaining soluble activable enzyme (*5*). The failure to form disulfide bonds, or their incorrect formation, appears to be characteristic of prochymosin produced in the cytoplasm, both in yeast (as we have shown) and in *E. coli* (*22*). Secretion of a fully activable proenzyme eliminates many of the processing steps required to isolate activable protein from the cytoplasm. Unfolding in denaturants and refolding, which is frequently inefficient in vitro, are not required. However, yeast cells have a limited capacity for prochymosin secretion. For example, a fivefold increase in production of prochymosin through use of a stronger promoter results in secretion of only one-tenth more prochymosin (experiments 5 and 6 in Table 1).

We have identified effective methods for reducing the limitations on secretion of prochymosin. The nature of the secretion signal is important because the native calf signal is only about one-fourth as efficient as the yeast signal from invertase. However, even with the invertase signal sequence, less than 5 percent of the prochymosin produced is secreted. Since secretion of prochymosin can be blocked either by the absence of additional signals required for yeast secretion or by the presence of signals that shunt it to intracellular compartments, we decided to alter the host cell function by mutation. This approach is effective not only with prochymosin but also with bovine growth hormone (BGH).

The simplest model to account for the supersecreting mutations is that they open new bypass routes around a single rate-limiting step in the secretion of heterologous proteins. This model is also consistent with two other characteristics of the mutants—additivity and protein specificity. Clearly, if the bypass pathway is still rate-limiting, additional alternative routes will improve the overall rate. At the same time, it is reasonable to assume that certain proteins will not flow through certain bypass pathways. The fact that BGH secretion is improved dramatically by the *ssc*1-1 mutations but only marginally by the *ssc*2-1 mutation could be an example of differential flow through the alternative routes.

At least two groups have reported isolation of mutants with altered localization of homologous yeast vacuolar proteins (*23*). However, the effects of those mutations on heterologous proteins have not been reported.

Finally, increased efficiency of both prochymosin gene expression and protein secretion has been observed from a surprising manipulation—integration of the transcriptional unit into the genome. The effect is significant and reproducible, but the mechanism is unclear; it may reflect differences in either the rate or the level of expression from plasmid-borne and integrated copies of the gene. We find that prochymosin production from plasmids does not respond in a linear fashion to increases in transcriptional unit copy number because the expression level from plasmids at more than 100 copies per cell is not 100 times but only about 10 times the level from a single-copy integrant (*14*). Transcriptional or translational limitations for some promoters may prevent a linear response to copy number. Whatever the explanation, the efficiency of expression per gene copy is much higher at low copy numbers.

The supersecreting mutants increase the secretion level for prochymosin produced from both integrated and plasmid-borne transcriptional units; however, they do not alter the nonlinear response between plasmid-borne gene dosage and expression. Furthermore, secretion of prochymosin made from plasmid copies of the gene appears to be saturated at a lower level than secretion of prochymosin made from integrated copies, even in *ssc*1 and *ssc*2 host strains. One possible explanation for this saturation is that gene expression from plasmids may be more synchronous than that from multiple, integrated gene copies dispersed throughout the genome. Thus, pulses of expression delivering saturating amounts of protein to the secretion pathway would overwhelm not only the rate-limiting step but also the putative bypass steps opened by the supersecreting mutations, causing a logjam effect.

In conclusion, secretion is necessary for production of activable prochymosin. By combining the benefits of yeast secretion signals, multiple integrated transcriptional units, and specific mutations of the host genome, we have increased secretion of prochymosin into the growth medium at least 80-fold, thereby allowing production of fully activable prochymosin at levels of 20 mg per liter of culture medium. The existence of techniques to improve yeast secretion of heterologous proteins should aid in the production of other mammalian secreted proteins in their native active conformations.

References and Notes

1. R. Schekman and P. Novick, in *The Molecular Biology of the Yeast Saccharomyces: Metabolism and Gene Expression*, J. N. Strathern, E. W. Jones, J. R. Broach, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982), pp. 361–393.
2. A. Singh, J. M. Lugovoy, W. J. Kohr, L. J. Perry, *Nucleic Acids Res.* **12**, 8927 (1984).
3. A. J. Brake *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4642 (1984).
4. G. A. Bitter, K. K. Chen, A. R. Banks, P. H. Lai, *ibid.*, p. 5330.
5. D. T. Moir, J. Mao, M. J. Duncan, R. A. Smith, T. Kohno, in *Developments in Industrial Microbiology*, L. Underkofler, Ed. (Society for Industrial Microbiology, 1985), vol. 26, pp. 75–85.
6. R. G. Schoner, L. F. Ellis, B. E. Schoner, *Biotechnology* **3**, 151 (1985).
7. D. Pennica *et al.*, *Nature (London)* **301**, 214 (1983).
8. G. Simons, E. Remaut, B. Allet, R. Devos, W. Fiers, *Gene* **28**, 55 (1984).
9. C. G. Goff *et al.*, *ibid.* **27**, 35 (1984).
10. J. S. Emtage *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3671 (1983).
11. F. A. O. Marston *et al.*, *Biotechnology* **2**, 800 (1984).
12. V. B. Pedersen and B. Foltmann, *Eur. J. Biochem.* **55**, 95 (1975).
13. D. Moir *et al.*, *Gene* **19**, 127 (1982).
14. M. J. Duncan and K. Stashenko, in preparation.

15. D. D. Pless and W. J. Lennarz, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 134 (1977).
16. R. B. Trimble and F. Maley, *J. Biol. Chem.* **252**, 4409 (1977).
17. B. Foltmann, *Methods Enzymol.* **19**, 421 (1970).
18. R. A. Smith and T. Gill, *J. Cell. Biochem.* (suppl. **9C**), 157 (1985).
19. _____, in preparation.
20. R. J. Summers and S. Roof, personal communication.
21. J. B. Hicks, A. Hinnen, G. R. Fink, *Cold Spring Harbor Symp. Quant. Biol.* **43**, 1305 (1979).
22. J. M. Schoemaker, A. H. Brasnett, F. A. O. Marston, *EMBO J.* **4**, 775 (1985).
23. S. D. Emr, V. A. Bankaitis, J. M. Garrett, M. G. Douglas, in *Protein Transport and Secretion*, M.-J. Gething, Ed. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1985), p. 184; J. H. Rothman *et al.*, *ibid.*, p. 190. Complementation tests show that the *vpt3* and *vpt5* mutants of Emr are different from *ssc1* and *ssc2*.
24. M. Carlson, R. Taussig, S. Kustu, D. Botstein, *Mol. Cell. Biol.* **3**, 439 (1983).
25. J. Kurjan and I. Herskowitz, *Cell* **30**, 933 (1982).
26. K. Arima *et al.*, *Nucleic Acids Res.* **11**, 1657 (1983).
27. T. Alber and G. Kawasaki, *J. Mol. Appl. Genet.* **1**, 419 (1982).
28. M. Carlson and D. Botstein, *Cell* **28**, 145 (1982).
29. M. Rose and D. Botstein, *J. Mol. Biol.* **170**, 883 (1983).
30. H. Towbin, T. Staehelin, J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350 (1979).
31. YNB contained, per liter: 7 g of yeast nitrogen base (Difco), 20 g of glucose or galactose, and 20 g of agar (when used in plates). Detection of active chymosin required the addition of bovine serum albumin (25 μg/ml) to liquid medium but not to agar plates. Acid activation was unnecessary, except when succinate-buffered YNB (YNB buffered at *p*H 6 with 10 g of succinic acid and 6 g of NaOH per liter) was used, in that yeast metabolism reduces the *p*H of the medium to levels sufficient to activate all the prochymosin produced.
32. K. Struhl, D. T. Stinchcomb, S. Scherer, R. W. Davis, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1035 (1979).
33. We thank T. Gill and K. Stashenko whose work was vital to the success of this project. We are grateful for key contributions from the following people at Collaborative Research, Inc.: J. Mao, T. Kohno, E. Yamasaki, M. E. Rhinehart, R. Knowlton, V. Brown, S. Porteous, and C. Stillman. We thank R. J. Summers and S. Roof of Dow Chemical Company for unpublished yeast fermentation results. Purified native calf prochymosin and rabbit antiserum directed against calf chymosin were gifts from R. Goltz of Dow Chemical Company. We thank G. Fink and G. Vovis for critically reading the manuscript. This work was partially supported by a contract with Dow Chemical Company.

Biotechnology in Food Production and Processing

Dietrich Knorr and Anthony J. Sinskey

Summary. The food processing industry is the oldest and largest industry using biotechnological processes. Further development of food products and processes based on biotechnology depends upon the improvement of existing processes, such as fermentation, immobilized biocatalyst technology, and production of additives and processing aids, as well as the development of new opportunities for food biotechnology. Improvements are needed in the characterization, safety, and quality control of food materials, in processing methods, in waste conversion and utilization processes, and in currently used food microorganism and tissue culture systems. Also needed are fundamental studies of the structure-function relationship of food materials and of the cell physiology and biochemistry of raw materials.

The use of biotechnology in the manufacture of food and beverages has been practiced for more than 8000 years with vinegar, alcoholic beverages, sourdough, and cheese production being the most prominent examples (*1*). Biotechnological processes are now being used to produce other fermented products, food and feed additives, and processing aids (Table 1). In fact, the food processing industry, which has annual sales of $300 billion in the United States and about £30 billion in Great Britain, is the oldest and largest user of biotechnological processes (*2*).

An important issue today is the impact that modern biology will have on the food industry. Recent advances in molecular biology, fermentation science, tissue culture systems, and bioengineering offer great potential for application to several areas of food production and processing. A key question related to this issue is, what are the constraints hindering applications of biotechnology to the food industry? In this article, we attempt to address these points and to review the current role of biotechnology in the production and processing of food.

Biotechnology in Food Production

Biotechnology can significantly influence the food supply, including the production and preservation of raw materials and the alteration of their nutritional and functional properties. In addition, development of production aids, processing aids, and direct additives such as enzymes, flavors, polysaccharides, pigments, and antioxidants can improve the overall utilization of raw materials.

Raw materials. Plant products derived from fewer than 30 plant species provide more than 90 percent of the human diet. Eight cereal crops supply more than half the world's calories (*4*). Animal products contribute over 56 million tons of edible protein and over 1 billion megacalories of energy annually (*5*). In addition, the increasing importance of marine food products and single cell proteins (SCP) as raw materials has been stressed (*6*).

Currently, the role of biotechnology in raw material production is directed toward (i) increasing productivity through improved efficiency of nutrient use and conversion, (ii) increasing productivity through improved plant resistance, and (iii) identifying new food sources with desirable properties.

Feed efficiency and productivity of animals has been increased substantially (*7*). Furthermore, SCP, derived from the dried cells of microorganisms for use as protein sources in human food and animal feeds, are cultivated on a large scale by using both photosynthetic and nonphotosynthetic microorganisms (*8*). Extensive work is under way to increase the ability of plants to fix atmospheric nitrogen for their metabolic use (*9*), and cultured plants and plant cells are being considered for food production (*10*).

Additional efforts include the genetic

Dietrich Knorr is professor of food processing and biotechnology, Biotechnology Group, Department of Food Science, University of Delaware, Newark 19716. Anthony J. Sinskey is professor of applied microbiology, Department of Applied Biological Sciences, Massachusetts Institute of Technology, Cambridge 02139. This article was adapted from a position paper presented at the Institute of Food Technologists workshop on research needs, 11 to 14 November 1984, Arlington Heights, Illinois, and from a subsequently developed document on research needs in food biotechnology.