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ISOLATION AND GENETIC ANALYSIS OF *Aspergillus niger* MUTANTS WITH REDUCED EXTRACELLULAR GLUCOAMYLASE

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ABSTRACT

Mutants with impaired production of extracellular glucoamylase were isolated at a high frequency (2% of survivors) from an *Aspergillus niger* strain treated with UV light. These were designated as low glucoamylase producers (*lgp*, up to 30% of the parental yield) and medium producers (*mgp*, a 35 to 50% decrease in enzyme level). All the mutants were shown to be recessive; one strain segregated two unlinked genes. Complementation tests, and segregation from heterozygous diploids, suggested at least three to four unlinked genes, each able to impair glucoamylase production. There is evidence of a single structural gene for glucoamylase in *A. niger*. Therefore, as production of extracellular enzymes is normally the final result of several steps at intracellular and membrane levels, including regulation of enzyme synthesis, we suggest intergenic interaction that controls extracellular enzyme accumulation and that mutation in any of these genes would result in impaired production.

INTRODUCTION

Glucoamylase (E.C.3.2.1.3.) is of economic interest because of its capacity to turn starch and related polymers into *B*-D-glucose as the sole end product (Banks *et al.*, 1976). This enzyme occurs almost exclusively in fungi and is produced at an industrial level by *Aspergillus* species. Of these, *A. niger* should be cited as one of the major producers (Fogarty and Kelly, 1980).

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Several studies have been carried out on fermentation and molecular aspects of enzyme production; they revealed that, although a single copy of the structural gene seems to exist in *A. niger*, two main forms of the enzyme can be found extracellularly (Svensson *et al.*, 1982; Boel *et al.*, 1984a,b). These two forms are essentially equally active towards soluble polysaccharide and oligosaccharide substrates, while the larger form, G1, possesses the capacity to adsorb onto and to digest raw starch (Svensson *et al.*, 1982). The occurrence of two forms was initially attributed to differential mRNA processing but there is now strong evidence of a limited, post-translational proteolysis of the G1 form to give at least two G2 forms (Svensson *et al.*, 1986).

The glucoamylase system is very useful because of the large amount of enzyme protein that can be accumulated extracellularly by highly mutated strains in appropriate conditions (Van Brunt, 1986; Saha and Zeikus, 1989). It has also stimulated interest in the use of transcription, translation and secretory control regions of this gene for heterologous gene expression (Cullen *et al.*, 1987; Gwynne *et al.*, 1987).

Nevertheless, there are few genetic studies on glucoamylase production (Chang and Terry, 1975; Ball *et al.*, 1978; Bonatelli Jr. *et al.*, 1984). Studies with *A. niger*, as well as with other species (Chang and Terry, 1975), have revealed that most auxotrophy or drug resistance mutations show pleiotropic effects on enzyme production. Such effects, though undesirable for genetic analysis, might, if confirmed, be used to determine rate-limiting steps in glucoamylase production other than those directly involved in enzyme polypeptide production (Masiero and Bonatelli Jr., 1989).

In this work we describe the isolation of mutants with impaired production of glucoamylase from *A. niger* strains, and the genetic studies that have been carried out to establish linkage relationships and allelic interactions of these mutants.

MATERIAL AND METHODS

Organisms

The *A. niger* strains used were: *pabA1*, *fwnA1* and *nicA1 olvA3* (Bonatelli Jr. *et al.*, 1982; Masiero and Bonatelli Jr., 1989). These mutant alleles determine: *fwnA1*, fawn conidia; *olvA3*, olive conidia; *nicA1* and *pabA1*, requirement for nicotinic acid and *p*-aminobenzoic acid, respectively.

Media

The growth media were those described by Pontecorvo *et al.* (1953) and, for glucoamylase production, the medium (MAC) described by Bonatelli Jr. *et al.* (1984)

supplemented when necessary according to nutritional requirements (Masiero and Bonatelli Jr., 1989).

Mutant induction and isolation

Conidia from 5-7 day old colonies were collected in Tween 80 solution (0.1%, v/v) and the suspension adjusted to 10^6/ml with saline (NaCl 0.85%, w/v). Treatments were with ultraviolet light (254 nm) to give 1-5% survival; treated conidia were plated on complete medium after dilution to yield 10-20 colonies per Petri dish. Plates were incubated up to four days at 28°C. Colonies were inspected visually and those showing a near-parental morphology (conidial color and colony size) were numbered, and a loopful of conidia from each was inoculated into test tubes containing 5 ml of MAC for a preliminary screening. These were incubated for four days at 28°C, together with a minimum of five replicates of the parent. Colonies showing reduced enzyme production were purified and at least four 125 ml Erlenmeyer flasks containing 25 ml of MAC were inoculated with 1 ml of a 10^6 conidia/ml suspension, five replicates of the parental strain were used as a control. Cultures were incubated for four days at 28°C; only those with a significantly reduced production were retained; for confirmation a further similar assay was carried out. Colonies were also assayed for any new auxotrophic characters, and only those showing parental strain auxotrophic markers were retained.

Enzyme assays

From test tubes (see above), 100 µl of the culture filtrate was incubated for 60 minutes, in a 60°C water bath, with 1 ml of a 1% soluble starch solution in 50 mM citrate buffer at pH 4.0, plus 900 µl of distilled sterilized water (Park and Papini, 1970; Banks *et al.*, 1976). Culture filtrates from Erlenmeyer flasks were appropriately diluted and 500 µl was mixed with an equal volume of the above soluble starch solution and incubated as previously described. Reducing sugars and glucose were quantified initially at the same time with *o*-toluidine and glucose oxidase reagent kits (BIOBRAS or DOLES). There was no significant difference between these estimates nor were the estimates obtained by additional tests with TAKA-DIASTASE limit dextrin and with a crude preparation of the glucoamylase inhibitor described by Ueda and Koba (1973) significantly different. It was apparent, therefore, that glucoamylase was the main product (> 80%). The *o*-toluidine reagent kit was used in most of that work. One unit of enzyme (U/ml) was as described previously (Bonatelli Jr. *et al.*, 1984; Masiero and Bonatelli Jr., 1989).

Isolation and characterization of diploids and segregants

The procedure was that described by Masiero and Bonatelli Jr. (1989) except that enzyme production was also assayed from a sample of up to 50 haploid segregants per diploid synthesized (usually 25 fawn and 25 olive segregants), as described for the preliminary screening of reduced production mutants.

Statistical analysis of the enzyme production of segregants

When the sample of segregants, previously characterized for conidial colour and nutritional markers, was assayed for enzyme production, 15 replicates of each parental strain were included as controls. The first analysis was carried out by comparing the enzyme production of parental and segregants with the Kolmogorov-Smirnov test (Siegel, 1975) to see if there was any distortion in the segregation of the parental and mutant alleles involved with enzyme yield. It was presumed that the production of the parental strain(s) (with wild type allele(s)) should be significantly different from that observed for the mutant strain(s) (with mutant allele(s)) and also that each will differ from the segregants. But if the distribution of production of the segregants was compared with a combined distribution of both parentals it should not differ unless there was segregation distortion. The second analysis was that used by MacDonald *et al.* (1972) for genetical analysis of mutations impairing penicillin production in *A. nidulans*, except that means were compared with the Kruskal-Wallis or Mann-Whitney U test (Siegel, 1975). The theoretical reasoning for that analysis is summarized elsewhere (MacDonald *et al.*, 1972).

RESULTS AND DISCUSSION

Strain *nicA$_1$ olvA$_3$* was treated with UV light and from the colonies that survived, 268 were assayed in a preliminary screening, with 32 replicates of the parental strain as controls (Figure 1). It can be seen that the parental strain mean, 2.9 Units/ml (U/ml), was almost equal to that observed for survivors, 2.5 U/ml, and that a considerable number of colonies produced less than 1U of enzyme/ml. Most were assayed again, after purification; eight of them (2.1%) showed a significantly impaired production and were retained (Table I). The same procedure was used for the *pabA$_1$ fwnA$_1$* strain (data not shown) and two out of 95 colonies (2.1%) were selected as impaired producers (Table I). Mutants with up to 30% of the parental yield were designated low glucoamylase producers (*lgp*) and those producing from 35 to 50% less were designated as *m*edium *g*lucoamylase

producers (*mgp*). Only one mutant, *lgp38*, showed morphological changes (poor conidiation and lower growth rate) after purification. None of the others had altered auxotrophic requirements, and this excludes easily-identifiable pleiotropic effects (Masiero and Bonatelli Jr., 1989). Although the frequency of enzyme-production mutants is not strictly comparable to the frequency of auxotrophic mutants, it is remarkable that the former is almost 10 times greater than the latter in strain *pabA1 fwnA1*, even after filtration enrichment (Masiero and Bonatelli Jr., 1989).

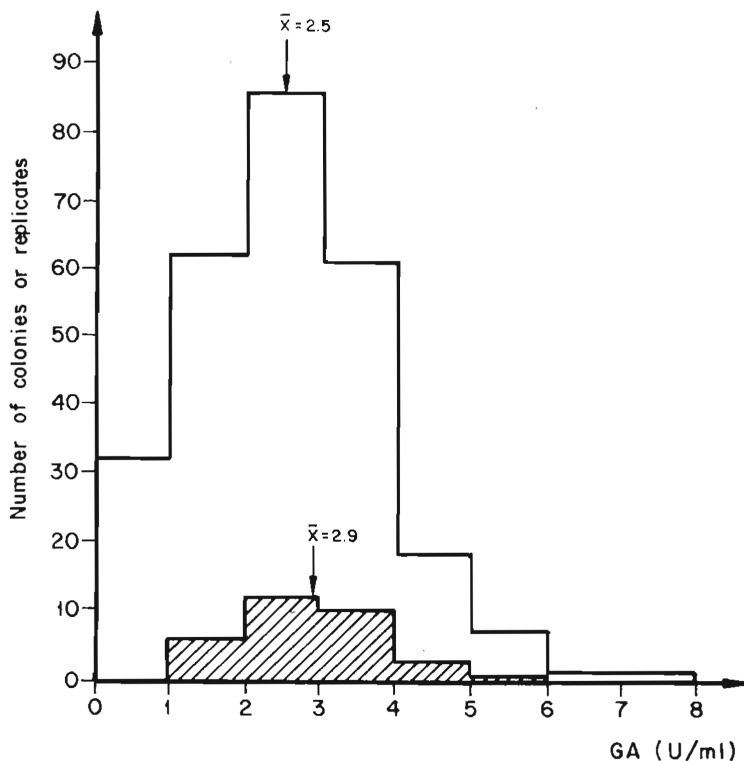

Figure 1 - Distribution of glucoamylase (GA) production of colonies obtained after UV light treatment compared to parental *nicA1 olvA3* (cross hatched area).

It seems reasonable to suggest, therefore, that a number of genes are involved in the production of extracellular glucoamylase. This possibility is reinforced by the fact that multiple steps of mutation and selection are usually required to achieve a high level

of enzyme (Nevalainen and Palva, 1979); as for other extracellular enzymes, yield is usually considered to be the result of many steps at intracellular and membrane levels, including regulation of enzyme synthesis (Ramaley, 1979; Eveleigh and Montenecourt, 1979).

Table I - Glucoamylase (GA) production from parental and mutant strains.

Strains*	GA production (U / ml) ± s.d.
$nicA_1$ $olvA_3$ (parental)	11.3 ± 2.8 (24)**
mgp3	7.0 ± 0.8 (5)
mgp100	5.9 ± 1.0 (9)
mgp111	6.5 ± 2.1 (5)
mgp166	7.2 ± 0.8 (9)
mgp199	6.1 ± 1.7 (5)
lgp38	2.0 ± 0.9 (13)
lgp73	1.5 ± 1.2 (15)
lgp33	0.0 ± 0.0 (5)
$pabA_1$ $fwnA_1$ (parental)	13.0 ± 1.4 (19)
lgp5	1.9 ± 0.6 (5)
lgp61	0.3 ± 0.6 (10)

* Parental strains followed by mutants derived from them.

** Number of replicates.

To test this hypothesis, via allelic interaction, a number of mutants were combined in diploids with an appropriate parental haploid. All of the mutations were recessive (Table II). Mutants *lgp38* and *lgp73* were also combined with *lgp61* as the formers bears auxotrophic and conidial colour markers complementary to the latter (Table I). Complementation for enzyme production was also observed (Table II); different genes were altered in these strains. Only heterokaryons were obtained when *lgp5* was combined with *$nicA_1$ $olvA_3$* or mutants derived from these strains (data not shown). Incompatibility appears to occur at the nuclear level, and it seems likely that only isolation of revertants of the *lgp* phenotype will show if that incompatibility is a pleiotropic effect or if another gene was also altered by UV light treatment. This incompatibility was also observed when the *lgp5* mutant was fused with *A. awamori* strains (Oliveira and Bonatelli Jr., data not published).

Table II - Mean glucoamylase (GA) production of heterozygous diploids compared to control diploids (mean of five replicates).

Diploids*	GA production (U/ml) ± s.d.
pabA1 fwnA1 // nicA1 olvA3 (control)	10.1 ± 0.8
lgp33 // pabA1 fwnA1	13.1 ± 1.8
mgp100 // pabA1 fwnA1	12.7 ± 1.6
lgp73 // pabA1 fwnA1	12.6 ± 2.2
lgp38 // lgp61	10.7 ± 1.5
mgp3 // pabA1 fwnA1	10.5 ± 2.3
lgp61 // nicA1 olvA3	10.3 ± 1.7
lgp73 // lgp61	9.2 ± 1.6

* For symbols of strains see Table I.

Genetic analysis provided confirmation that several genes are involved in glucoamylase production. Results from the diploid *lgp61 // nicA1 olvA3* are given in detail; for the others, only relevant data are presented and discussed. From the former diploid, 50 haploid segregants were isolated and assayed for enzyme production together with 15 replicates of each parental strain. The distribution of *lgp61* production is quite different from that observed for *nicA1 olvA3*, and both differ from the segregants (Figure 2). Analysis with the Kolmogorov-Smirnov test (Siegel, 1975) confirmed this visual observation and also indicated that there is no difference between the enzyme production of segregants and that obtained from both parentals combined, this suggested that there was no apparent segregation distortion of *lgp61* and *lgp61*$^+$ phenotypes (Table III). It was possible also to assign a low production phenotype label, from now on attributed to the *lgpA61* gene, to linkage group II, which carries the *pabA* gene. Segregants with the *pabA* allele produced significantly less (0.9 U/ml) than those with the *pabA*$^+$ allele (4.0 U/ml) and production of segregants with alternative alleles on linkage group I did not differ (Table IV, MacDonald *et al.*, 1972).

It was also observed that *lgp73* was unable to grow on medium containing starch as the sole carbon source (Masiero, data not shown). At first this was considered to be a pleiotropic effect of the gene involved with glucoamylase production. However, segregation from two different diploids (see one of them in Tables V and VI) showed two unlinked genes which gave a ratio of three low (47) producers to one normal (7) producer. One of these genes, now designated *lgpB73*, decreased enzyme production up to three units; *lgpB73* is not carried in any of the linkage groups so far identified (Masiero and Bonatelli Jr., 1989; Masiero, not published data). The other, now designated *snuA1* (starch

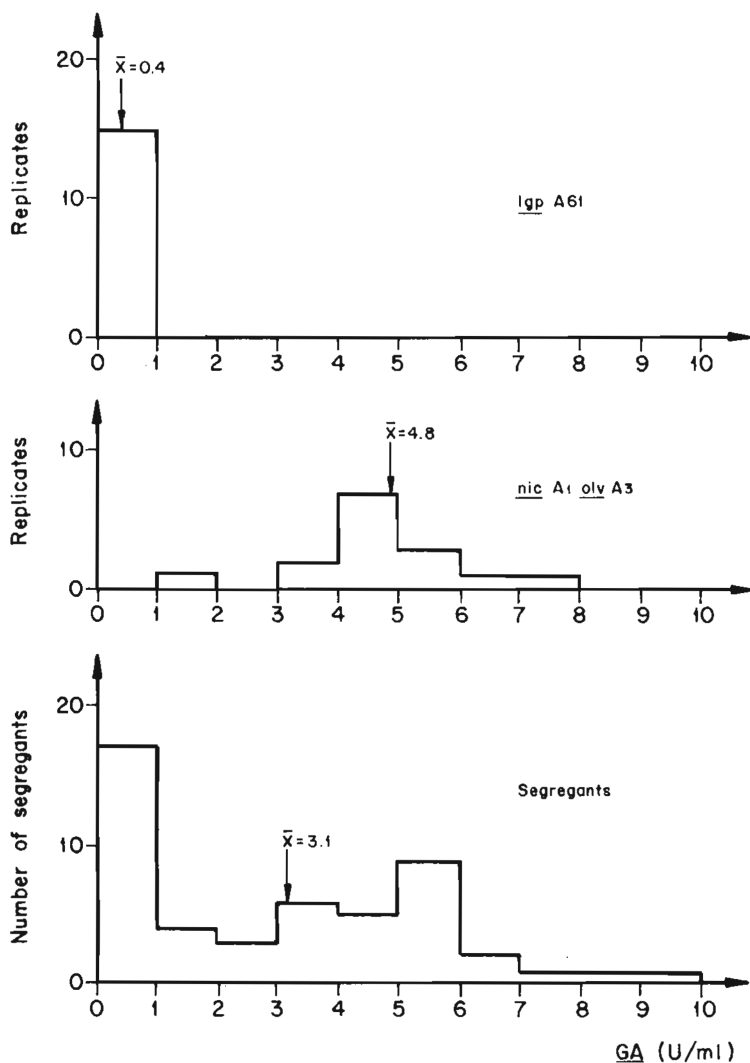

Figure 2 - Distribution of glucoamylase (GA) production of parental and haploid segregants from diploid *lgp61 // nicA1 olvA3*.

*n*on-*u*tilizing), is responsible for the inability to grow on soluble starch as a sole carbon source; but it allows normal growth on maltose or glucose, indicating that products of starch hydrolysis can be metabolized. However, enzyme production in maltose medium is similar to that observed on starch medium (Masiero, data not shown). One hypothesis,

currently under investigation, presumes that such a mutation can alter the activities of enzymes capable of starch hydrolysis, i.e. glucoamylase and alpha-amylase. The latter is produced at very low levels by the *A. niger* strain used (Bonatelli Jr. *et al.*, 1984) but probably allows strains with partially or totally blocked enzyme activity to grow as well on starch as wild type strains. Another interesting effect of *snuA1* is that it seems to decrease glucoamylase production even further when combined with *lgpB73*, as has been observed in the original *lgp73* strain (Table I). As *snuA1* is not carried in the linkage groups already identified in that strain, it is suggested that two further linkage groups have been detected in addition to the four described previously (Masiero and Bonatelli Jr., 1989).

Table III - Comparison of parental strains and segregants from diploid *lgp61 // nicA1 olvA3*.

Strains	Strains	
	nicA1 olvA3	Segregants
nicA1 olvA3	-	$0.4013^{+} < 0.4231^{*\,++}$
lgp61	0.4966 < 1.000*	0.4013 < 0.6531*
(*nicA1 olvA3* and *lgp61*)+++	-	$0.3153 > 0.1531^{ns}$

+, Values calculated for 5% significance level.

++, Values calculated comparing distributions of strains or strains with segregants.

+++, Combination of parental strains distribution.

ns, Non significant; *, Significant at 5% level (Kolmogorov Smirnov test).

Table IV - Mean glucoamylase (GA) production of haploid segregants from diploid *lgp61 //nicA1 olvA3*.

Linkage group	Marker segregating	Segregants with				Significance
		Mutant Allele		Wild Type Allele		
		no.	mean (U/ml)	no.	mean (U/ml)	
I	*nicA1*	22	3.3	27	2.9	1.8^{ns}
I	*olvA3*	23	3.3	26	2.9	1.8^{ns}
II	*pabA1*	14	0.9	35	4.0	15.2^{***}

ns, Non significant.

***, Significant at 0.1% level.

Table V - Comparison of parental strains and segregants from diploid *lgp73 // pabA1 fwnA1*.

Strains	Strains	
	lgp73	Segregants
lgp73	-	$0.4711 > 0.100^{ns}$
pabA1 fwnA1	$0.608 < 1.000^{*}$	$0.4711 < 0.940^{*}$
(*lgp73* and *pabA1 fwnA1*)	-	$0.3598 < 0.440^{*}$

ns, Non significant.

*, Significant at 5% level, (Kolmogorov-Smirnov test).

(for more details see Table III).

Table VI - Mean glucoamylase (GA) production of haploid segregants from diploid *lgp73 // pabA1 fwnA1*.

Linkage group	Marker segregating	Segregants with				Significance
		Mutant Allele		Wild Type Allele		
		no.	mean (U/ml)	no.	mean (U/ml)	
I	*nicA1*	21	1.1	29	0.1	0.537^{ns}
I	*olvA3*	22	1.1	28	0.1	0.305^{ns}
II	*pabA1*	28	0.3	22	0.9	0.098^{ns}

ns, Non significant.

Two mutants, *lgp38* and *mgp3*, were shown to have a low enzyme production phenotype, whose genetic determinant was not associated with linkage groups I or II. In the former, no low enzyme production or morphologically altered segregants were observed in a sample of 50 segregants; this suggested that these phenotypes could be traced either to one gene or to one linkage group (Table VII). Also, because of the enzyme production level, it would be reasonable to speculate that a further gene could be involved with glucoamylase production, in addition to those identified up to now in this work. Further investigations were not done with *mgp3*. As that strain seemed to have been lost before stock preparations, since typical *mgp3* production was not recovered (data not shown).

Table VII - Comparison of parental strains and segregants from diploid *lgp38//*$pabA_1$ $fwnA_1$.

Strains	Strains	
	$pabA_1$ $fwnA_1$	Segregants
$pabA_1$ $fwnA_1$	-	$0.4797 > 0.2366^{ns}$
lgp38	$0.4313 < 0.5212^{*}$	$0.4797 < 0.8310^{*}$
($pabA_1$ $fwnA_1$ and *lgp38*)	-	$0.4556 < 0.5364^{*}$

ns, Non significant; *, Significant at 5% level, (Kolmogorov-Smirnov test).
(for more details see Table III).

We suggest that there are at least 3-4 genes which can markedly affect glucoamylase production in *A. niger* and that they are distributed throughout the genome. As there is evidence of a single copy of the structural gene (Boel *et al.*, 1984a,b) we suppose that production can be considerably affected by mutation of gene(s) other than the structural genes. We also think that these genes exert their action on regulation or secretion, which influence extracellular enzyme accumulation. If one of these is affected by a mutation, production is impaired. Several groups are interested in heterologous protein production using glucoamylase control regions (Cullen *et al.*, 1987; Gwynne *et al.*, 1987; Contreras *et al.*, 1991; Ward, 1991). The gene interaction detected in this work might also be considered for industrial level production.

On the basis of these results work is now in progress along three main lines. The first is a study of complementation with some of the other mutants, to isolate recombinants with two mutant genes and to establish linkage relationships, using master strains constructed by Bos *et al.* (1988) and Debets (1990), as our strain is apparently compatible with the latter (data not shown). The second involves complementation of *lgp* mutations in interspecific hybrids with *A. awamori*. The third aims to establish conditions of high frequency of transformation for our strain, in order to isolate functional DNA sequences by complementation of mutants. This has been done with *A. nidulans* (Ballance and Turner, 1986). More recently, a possibility of doing this with *A. niger* was advanced by Campbell *et al.* (1989). Another approach to identify which function (secretion or regulation) is affected in these mutants is to determine intracellular enzyme levels. This work is now in progress.

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RESUMO

Mutantes com redução na produção de glicoamilase extracelular foram isolados com alta frequência (2%) de uma linhagem de *Aspergillus niger*. Estes foram designados de baixa produção de glicoamilase (*lgp*) quando produziram até 30% do valor observado nas parentais e, média produção de glicoamilase (*mgp*), quando mostraram redução de 35 a 50%. Todos os mutantes estudados foram recessivos e a linhagem *lgp73* segregou 2 genes não ligados. Testes de complementação e segregação de diplóides heterozigotos indicaram 3 a 4 genes em diferentes grupos de ligação que afetam consideravelmente a produção da enzima. Considerando a evidência de uma única cópia do gene estrutural nesta espécie e que, produção de enzimas extracelulares como é o caso, é normalmente o resultado final de vários passos a nível intracelular e de membrana incluindo regulação, parece razoável sugerir que haja interação gênica no acúmulo desta enzima e que, mutação em qualquer um destes genes, deve resultar em diminuição da produção.

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