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Amylolytic enzymes produced by the yeast *Saccharomycopsis fibuligera*

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Two strains of the food-borne amylolytic yeast *Saccharomycopsis fibuligera* were selected from a broad spectrum of *S. fibuligera* strains found in culture collections of microorganisms. These were analysed with respect to production and characterisation of their amylolytic enzymes. *S. fibuligera* KZ represents a strain synthesizing an amylolytic complex composed of α-amylase, glucoamylase and α-glucosidase. *S. fibuligera* IFO 0111 represents a strain producing only one amylolytic enzyme -glucoamylase, with a property unique among yeast amylases, namely the ability to degrade raw starch. Information on molecular-genetic aspects and enzymatic behaviour of amylolytic enzymes produced by both strains is presented.

Key words: *Saccharomycopsis fibuligera*, α-amylase, glucoamylase, α-glucosidase, raw starch degradation.

Abbreviations: Gla, Glu and Glm, glucoamylases synthesized by *S. fibuligera* KZ, HUT7212 and IFO 0111, respectively; SBD, starch-binding domain.

Introduction

Starch constitutes the most abundant rapidly-renewable source of energy for living organisms. This heterogeneous polysaccharide, composed of two high-molecular-weight components: linear amylose (α-1,4-linked D-glucose residues) and branched amylopectin (containing both α-1,4- and α-1,6-linked D-glucose residues), is degraded predominantly by hydrolytic enzymes called amylolytic enzymes. A broad variety of organisms, among them yeasts, are producers of these enzymes. More than 150 yeast species can degrade starch (Mc Cann & Barnett, 1986). Although amylases produced by yeasts do not have the wide application of those produced by bacteria and other fungi, there are amylases from yeasts such as *Saccharomycopsis fibuligera,* that are currently exploited in starch saccharification during food fermentation (Kato et al., 1976). *S. fibuligera* is a food-borne, dimorphous yeast, which has been considered, in the realm of ascomycetous yeast species, as one of the best producers of amylolytic enzymes (De Mot et al., 1984). The capability of *S. fibuligera* to degrade starch is connected with the production of two types of amylases: endo-acting α-amylase and exo-acting glucoamylase. Some *S. fibuligera* strains synthesize both enzymes while others produce only one type of amylase. In a few *S. fibuligera* strains, other en-

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Table 1. Enzymatic properties of α-amylase, α-glucosidases and glucoamylases from strains *Saccharomycopsis fibuligera* KZ and IFO 0111.

	KZ				IFO0111
	α-amylase	α-glucosidase extracellular	α-glucosidase cell-associated	glucoamylase Gla	glucoamylase Glm
Molecular weight[a]	54	132–135	132–135	62	55
pH optimum	5–6.2	5.5	5.5	5–6	5.5
T_{opt} [b]	40–50	52.5	42.5	40–50	40
Residual activity[c]	3	0	0	55	0
Raw starch digestion[d]	nd	–	–	–	+

[a] Molecular weight in kDa, [b] T_{opt}: Temperature optimum in °C, [c] Residual activity after 10 min. boiling in %, [d] nd – not determined.

zymes with specificity for α-D-glucosidic linkages, i.e. mainly α-glucosidases and transglucosylases, were detected (KOLTSOVA & SADOVA, 1970). The ability of amylases to digest raw starch is a technologically interesting property. Such enzymes can be used for energy saving in starch-processing (SAHA & UEDA, 1983). Raw starch degradation is rare among yeast amylases, but one of the known yeast enzymes having this capability is the glucoamylase produced by *S. fibuligera* IFO 0111.

To pursue our interest in using *S. fibuligera* as a donor of genes coding for commercially-interesting amylases we decided to analyse in detail amylases produced by two strains: *S. fibuligera* KZ, because of the capability of the amylases forming its amylolytic complex to renew enzymatic activity after thermal denaturation and *S. fibuligera* IFO 0111, because of the ability of its glucoamylase to digest raw starch. Glucoamylases produced by both strains are for us now the object of basic research in the field of structural biology. The strains have been further analysed for potential commercial production of amylases on the basis of environmentally-friendly technologies. In this contribution we summarise information on molecular-genetic and enzymatic characterisation of amylases produced by both strains.

Saccharomycopsis fibuligera KZ

The strain was obtained from the Institute of Food Technology, Vienna, Austria. Originally it was used in a one-step fermentation procedure for biomass production on waste starchy substrates (POLÍVKA & ZELINKA, 1969). Three types of enzymes hydrolysing α-1,4 glucosidic linkages are produced by this strain: α-amylase, glucoamylase and α-glucosidase (GAŠPERÍK et al., 1988; GAŠPERÍK & HOSTINOVÁ, 1990). The extracellular amylolytic complex was found to retain 45% of its original amylase activity after 10 min incubation at 100 °C and, therefore, we further analysed individual components of the complex mainly with focus on heat inactivation.

α-Amylase

The α-amylase is a glycoprotein, secreted into the extracelllular medium in multiple forms. Isoenzymes with identical enzymatic properties were isolated from a complex of extracellular amylolytic enzymes (GAŠPERÍK et al., 1991). The enzymatic characteristics of the α-amylase are given in Table 1. The gene coding for the α-amylase was isolated from the genomic DNA library containing 40 000 clones by a direct expression in *Saccharomyces cerevisiae* (HOSTINOVÁ et al., 1990). The gene and its gene product were not further studied in detail.

α-Glucosidase

α-Glucosidase was a component of the extracellular amylolytic complex of *S. fibuligera* KZ (GAŠPERÍK & HOSTINOVÁ, 1990). Later, α-glucosidase was also found in a cell-wall-associated form. High levels of the cell-wall-associated and extracellular α-glucosidases were synthesized on a medium containing cellobiose as a sole source of carbon (REISER & GAŠPERÍK, 1995). Both enzymes were purified. Their enzyme characteristics are presented in Table 1. The amino-acid sequence of six peptides from the extracellular and cell-wall-associated enzymes, prepared from different parts of the polypeptide chains showed a significant similarity to those of *Schwanniomyces occidentalis* glucoamylase (DOHMER et al., 1990). On the basis of these results we concluded that α-glucosidase from *S. fibuligera* KZ is an α-glucan hydrolase belonging to the family 31 of the glycoside hydrolase classification based on amino acid sequence

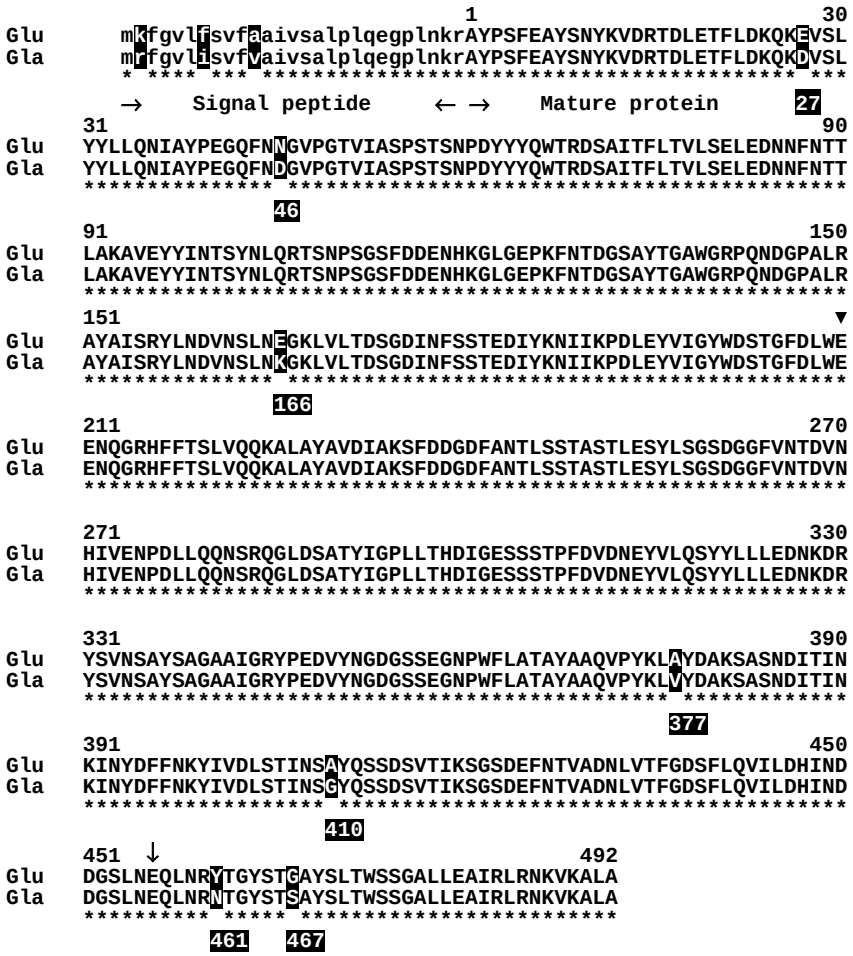

Fig. 1. Amino-acid alignment of glucoamylases Glu and Gla. The amino-acid sequence of the glucoamylases Glu and Gla was predicted from the nucleotide sequence of the *GLU* (Itoh et al., 1987) and *GLA* (Hostinová et al., 1991) genes. The small letters represent amino-acid residues in the signal peptide region. The capital letters represent amino-acid residues in the mature protein. The catalytic acids and bases are marked with the triangle and arrow, respectively. Identical amino-acid residues are marked with asterisks. Black boxes represent alterations in amino-acid residues.

similarities (Henrissat & Bairoch, 1993). The three-dimensional structure of proteins belonging to this family has not been determined yet.

Glucoamylase

Glucoamylase Gla is an extracellular glycoprotein which exists in multiple forms (Gašperík et al., 1991). Carbohydrate moieties, N-glycosidically-linked through mannose to asparagine residues, are responsible for this diversity (Gašperík & Hostinová, 1993). The role of the carbohydrate moiety in structure-function relationships has not been adequately examined yet. The enzyme characteristics of glucoamylase Gla are presented in Table 1. As can be seen from these data glucoamylase Gla has the highest ability to retain its catalytic activity after boiling compared to the other analysed enzymes. The glucoamylase gene *GLA* was isolated by a direct expressional cloning from the same genomic library as the α-amylase gene (Hostinová et al., 1990). Alignment of the nucleotide sequences of the *GLA* gene to the gene *GLU* isolated from the strain *S. fibuligera* HUT7212 (Yamashita et al., 1985) showed a high homology. Alignment of amino-acid sequences (Fig. 1) deduced from both genes (Itoh et al. 1987; Hostinová et al., 1991); re-

vealed seven amino acid alterations in the 492-residue-long mature polypeptide chain, which led to differences in specific activities and thermal stabilities between the Glu and Gla enzymes (GAŠPERÍK & HOSTINOVÁ, 1993). Except for the serine 467 in glucoamylase Gla, which was altered to glycine in glucoamylase Glu, all variant amino acids were localised outside the highly conserved regions of the different glucoamylases (ITOH et al., 1987; COUTINHO & REILLY, 1997). The mutation Gly467→Ser in glucoamylase Glu led to a decrease of k_{cat} to a value comparable to that of the Gla enzyme. Moreover, the mutant glucoamylase appeared to be less stable compared to the wild-type glucoamylase Glu (SOLOVICOVÁ et al., 1999). The tertiary structure was determined only for glucoamylase Glu (ŠEVČÍK et al., 1998). Crystals obtained from glucoamylase Gla were not suitable for diffraction analysis.

It is worth mentioning that the N-terminal amino-acid sequence of the first 20 amino acid residues of a recently isolated glucoamylase from *Saccharomycopsis* sp. TJ-1 corresponds to the sequence of the Gla glucoamylase (SUKARA et al., 1998). It would be interesting to know whether the primary structure of the entire polypeptide chain of glucoamylase from the TJ-1 strain is identical to Gla glucoamylase. The two strains seem to be different. While the producer of Gla synthesises the complex of several amylolytic enzymes, in the TJ-1 strain only glucoamylase was found (SUKARA et al., 1998).

Saccharomycopsis fibuligera IFO 0111

This strain is the only one, among *S. fibuligera* strains and other yeast species, known to produce a raw-starch-degrading amylolytic enzyme. *S. fibuligera* IFO 0111 produces glucoamylase Glm. Synthesis of other α-glucan hydrolases was not detected. Enzymatic properties of glucoamylase Glm are slightly different from glucoamylase Gla (Tab. 1). The enzyme Glm seems to be secreted only in one glycosylated form. The amino-acid composition of glucoamylase Glm compared to glucoamylase Gla is presented in Table 2. The ability of glucoamylase Glm to hydrolyse granular starch is very high and is comparable with that of *Aspergillus* and *Rhizopus* glucoamylases (UEDA & SAHA, 1983). Generally, the starch-binding domain (SBD) is responsible for raw starch digestion by amylolytic enzymes. In the majority of glucoamylases possessing a SBD this is located in the C-terminal (for a review, see SAUER et al., 2000), or in few enzymes in the N-terminal (BUI et al., 1996; ASHIKARI et al., 1986) part of a polypeptide chain and is separated from the catalytic domain. Enzymes from which SBDs have been removed have unchanged hydrolytic rates against soluble substrates (SVENSSON et al., 1982). The primary structure of glucoamylase Glm deduced from the gene (E. HOSTINOVÁ, unpublished data) shows that Glm lacks a separate SBD and that its raw-starch-affinity site/s are located within the intact enzyme. Glm glucoamylase is thus an interesting enzyme for structure-function relation studies at the level of the tertiary structure. New technological aspects of the application Glm are expected from such studies.

Table 2. Amino acid composition of glucoamylases Gla and Glm.

Residue	Gla	Glm
Ala	35	36
Arg	13	12
Asn	41	39
Asp	45	41
Cys	0	0
Gln	17	19
Glu	22	17
Gly	33	34
His	5	11
Ile	24	24
Leu	45	48
Lys	20	27
Met	0	0
Phe	21	19
Pro	16	18
Ser	54	51
Thr	33	32
Trp	6	10
Tyr	36	36
Val	26	25

Although the yeast *S. fibuligera* is a good producer of amylases with industrially interesting properties like raw starch degradation by glucoamylase Glm or the capability of glucoamylase Gla to retain the enzyme activity after boiling, they cannot compete with amylases produced commercially by bacteria and fungi. They can, however, be produced in applications like production of single-cell protein or ethanol from starchy biomass. The combined production of single-cell protein and amylase on waste starchy substrates has already been successfully tested (GAŠPERÍK et al., 1985). Thus, amylases from *S. fibuligera* are interesting not only as models for molecular-biological research but also for commercial applications.

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