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Expression, purification and preliminary crystallographic studies of α-amylase isozyme 1 from barley seeds

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The germinating barley seed contains two major α-amylase isozyme families, AMY1 and AMY2, involved in starch degradation to provide energy used by the plant embryo for growth. Many years of difficulty in growing three-dimensional crystals of natural AMY1 have now been overcome by a nonapeptide truncation of the enzyme C-terminus. The truncated enzyme was overexpressed in *Pichia pastoris*, purified and crystallized by the hanging-drop vapour-diffusion method using polyethylene glycol 8000 as precipitant and 2-propanol as an additive. Crystals belong to the orthorhombic space group $P2_12_12$, with unit-cell parameters $a = 88.36$, $b = 72.82$, $c = 61.74$ Å and one molecule per asymmetric unit.

1. Introduction

α-Amylases (1,4-α-D-glucan glucanohydrolases; EC 3.2.1.1) are monomeric enzymes widely occurring among animals, higher plants, fungi and micro-organisms. They catalyse the hydrolysis of internal α-D-1,4-glucosidic linkages in starch (amylose and amylopectin), glycogen and related oligosaccharides.

In barley, the physiological role of α-amylases is to secure the energy supply to the plantlet for growth. Two isozymes are distinguished in germinating seeds and are encoded by two multigene families. These isozymes, known as α-amylase 1 (AMY1) and α-amylase 2 (AMY2), contain 414 residues (MW = 45 342 Da) and 403 amino-acids (MW = 45 005 Da) (Rogers & Milliman, 1983; Rogers, 1985*a*), respectively, and share about 80% sequence identity, but show low overall sequence similarity to α-amylases from micro-organisms and animals (Rogers, 1985*b*).

AMY1 and AMY2 have different isoelectric points and are referred to as the low-pI and the high-pI isozyme, respectively (Jacobsen & Higgins, 1982). They differ significantly in their affinity for calcium ions (Bertoft *et al.*, 1984; Bush *et al.*, 1989; Rodenburg *et al.*, 1994) and in their stability at acidic pH (Rodenburg *et al.*, 1994) as well as at elevated temperature (Bertoft *et al.*, 1984). Moreover, large dissimilarities are found in their activity towards starch granules (MacGregor & Ballance, 1980; MacGregor & Morgan, 1986) and in their affinity for soluble substrates (Bertoft *et al.*, 1984; Søgaard & Svensson, 1990; Ajandouz *et al.*, 1992; MacGregor *et al.*, 1994).

Finally, an outstanding behaviour distinguishes AMY1 and AMY2: their sensitivity to the endogenous bifunctional inhibitor BASI (barley α-amylase/subtilisin inhibitor; Mundy *et al.*, 1983; Svendsen *et al.*, 1986; Leah & Mundy, 1989). While BASI is an excellent inhibitor for AMY2, with a K_i of 2.2×10^{-10} *M* at pH 8 and 310 K (Abe *et al.*, 1993; Sidenius *et al.*, 1995), it does not interact with AMY1. Both structural and biochemical studies have explained these differences (Vallée *et al.*, 1998; Rodenburg *et al.*, 2000).

Determination of the three-dimensional structure of AMY1 represents enormous progress in gaining insight into the major as well as the subtle differences between the two isozymes; AMY1 and AMY2 are thus the most well described α-amylase isozymes to date. In addition, studies of mosaic chimeras of AMY1 and AMY2 enable correlation of specific structural differences with specific enzymic and stability properties (Juge *et al.*, 1993, 1995; Rodenburg *et al.*, 1994, 2000). The crystal structure of AMY2 has previously been solved to 2.8 Å resolution (Kadziola *et al.*, 1994). Owing to large difficulties over more than a decade in growing AMY1 crystals suitable for X-ray diffraction, the gene encoding a truncated protein was constructed and expressed in a *P. pastoris* system established for the full-length AMY1 (Juge *et al.*, 1996). The recombinant AMY1 was subsequently crystallized and these crystals diffracted X-rays to 1.5 Å resolution.

2. Materials and methods

2.1. Construction of expression plasmid, recombinant production and purification

2.1.1. Strains and plasmids. *Escherichia coli* DH5α was used to propagate pBAL7ΔSI,II, which contains the wild-type insert encoding

AMY1 corresponding to that used previously (Matsui & Svensson, 1997). The silent mutations of the AMY1 gene removed *Sac*II and *Sac*I sites at positions 471 and 646 (Matsui & Svensson, 1997), making the remaining unique *Sac*I and *Sac*II sites useful for subcloning. *P. pastoris* GS115 (Invitrogen, The Netherlands) was used for expression of both the truncated AMY1 gene (see below) and the full-length gene inserted in pHIL-D2 (Invitrogen) as described previously (Juge *et al.*, 1996). Standard media were used for growth of *P. pastoris* transformants (Juge *et al.*, 1996).

2.1.2. Truncation of the AMY1 gene. Standard cloning techniques were used throughout this study (Gough & Murray, 1983; Sambrook *et al.*, 1989). Truncation of the AMY1 encoding sequence present in pBAL7ΔSI,II was performed by PCR using a downstream primer (17676), resulting in a new C-terminal residue that aligns to the C-terminus of the slightly shorter AMY2 [primer 17676: 5′-GCC GTC TGG GAG AAG AAC TGA *GAA TTC* GGA T**CC GCG G**CC GC, corresponding to AVWEKN followed by *Eco*RI (italic), *Bam*HI (underlined) and *Sac*II (bold) sites].

The upstream primer (5479) corresponds to the N-terminal sequence encoding the AMY1 signal sequence [primer 5479: 5′-TTT TTT *GAA TTC* ATG GGG AAG AAC GGC AGC CTG TGC, corresponding to *Eco*RI (italic) followed by MGKNGSLC].

The PCR product (∼1500 bp) encoded the C-terminally truncated AMY1 (Fig. 1). For amplification, Vent DNA polymerase (a high-fidelity thermophilic DNA polymerase, New England Biolabs, Beverly, USA) was used according to the manufacturer's instructions. The standard PCR consisted of 30 cycles at 367 (1 min), 328 (1 min) and 345 K (2 min). The PCR product was purified (Ultrafree MC, Pharmacia), subcloned in pHIL-D2 at the *Eco*RI site to yield pHIL-AMY1Δ9 and used for *E. coli* transformation. Single *E. coli* transformant colonies from agar plates were grown overnight in 5 ml LB/Amp (50 µg ml^{-1}), plasmids were purified (Plasmid Kit, Qiagen, Hilden, Germany) and the mutation was verified by DNA sequencing (Applied Biosystems 373A DNA Sequencer; Taq DyeDeoxy Terminator Cycle Sequencing Kit, Perkin–Elmer).

2.1.3. Transformation of *P. pastoris*. The yeast *P. pastoris* GS115 was transformed by electroporation according to the manufacturer's instructions (Invitrogen, Groningen, The Netherlands) with the expression plasmid pHIL-AMY1Δ9 which encodes the truncated AMY1, as inserted into pHIL-D2 at the unique *Eco*RI site between the AOX promoter and terminator (Juge *et al.*, 1996). His$^+$, Mut$^\div$ (methanol-utilization deficient) transformants were selected on His$^\div$ plates followed by plates with methanol as the sole carbon source to identify Mut$^\div$ colonies that indicated integration at the genomic AOX site. These transformants were tested for secretion of active enzyme on plates containing both starch and methanol (Gottschalk *et al.*, 2001).

2.1.4. Enzyme production. Fermentation of *P. pastoris* and production of the C-terminally truncated AMY1Δ9 were essentially performed as described by Juge *et al.* (1996). To monitor the secretion of active α-amylase variants, culture aliquots were removed during growth and assayed for activity toward Blue Starch (6.25 mg ml^{-1}) as described in Juge *et al.* (1995). AMY1Δ9 was purified from the concentrated culture supernatant by affinity chromatography on β-cyclodextrin Sepharose (Søgaard & Svensson, 1990; Juge *et al.*, 1996; Matsui & Svensson, 1997). The obtained AMY1Δ9 was analyzed (40–360 ng) using IEF (PhastGel, pI 4–6.5; Phast-System, Pharmacia), essentially as described in Søgaard *et al.* (1991) and Matsui & Svensson (1997). The enzyme was visualized by silver-staining and by a zymogram procedure involving soaking with a starch solution followed by addition of a KI/I_2 solution (Søgaard *et al.*, 1991) as well as by SDS–PAGE (PhastGel, 10–15%) and Western blotting (ProtoBlot Western Blot AP System, Promega, Madison, WI, USA) using 750-fold diluted anti-AMY2 immune serum (Matsui & Svensson, 1997). Enzyme concentrations were calculated from the amino-acid contents of protein (100 ng) hydrolysates (Alpha Plus amino-acid analyser, OPA-detection, Pharmacia) and secretion levels were assessed from the amounts of purified enzymes. The protein samples were concentrated (Centricon) and typically stored in 20 m*M* MES pH 6.7, 100 m*M* $CaCl_2$.

2.2. Activity assays

2.2.1. Insoluble Blue Starch. One 'Phadebas Amylase Test 100' tablet (Pharmacia) in 20 m*M* sodium acetate, 1 m*M* $CaCl_2$ pH 5.5 (4 ml) was added to AMY1 to a final concentration of approximately 2 n*M* and incubated for 15 min at 310 K. The reaction was stopped with 0.5 *M* NaOH (1 ml), 1 ml aliquots were centrifuged and the supernatants (300 µl) were transferred to a microtitre plate for A_{620} reading (Ceres UV900 HDI microplate reader, Biotek Instruments Inc.). Alternatively, 6.25 mg ml^{-1} insoluble Blue Starch (custom preparation, Pharmacia) was used in the above buffer containing 0.05% BSA (Matsui & Svensson, 1997). One unit of activity corresponds to the amount of enzyme giving ΔA_{620} = 0.001 in 15 min in the final volume (5 ml).

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AMY1 No.   397                ↓                414
AMY1 seq.    N D Y A V W E K N G A A A T L Q R S
AMY2 seq.    N D Y A V W E K I
AMY2 No.   395                403
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Figure 1
Comparison of the C-terminal sequences of barley α-amylase 1 and α-amylase 2. The arrow indicates the new C-terminal (Asn) of the truncated α-amylase 1.

2.2.2. Rationale for truncating the sequence. AMY1 and AMY2 have approximately 80% sequence identity (Rogers & Milliman, 1983; Rogers, 1985*a*). Despite this high identity, it has only been possible to obtain crystals of AMY2 that are suitable for X-ray studies (Svensson *et al.*, 1987; Kadziola *et al.*, 1994). Sequence comparison of the two proteins revealed a major difference between the polypeptide chains in the length of the C-terminal segment. AMY1 has a C-terminal extension nine amino-acid residues longer than AMY2. Furthermore, this C-terminal extension has been shown to be post-translationally modified in both barley and yeast (Søgaard *et al.*, 1991, 1993). Post-translational modifications such as proteolytic removal of C-terminal Arg-Ser by Kex1p, *O*-glycosylation and removal of additional C-terminal residues resulted in a heterogeneous mixture (Søgaard *et al.*, 1991, 1993) of AMY1 forms, which may be responsible for the failure to obtain good AMY1 crystals.

2.2.3. Production of AMY1Δ9. As *P. pastoris* secretes 20–50 mg l^{-1} wild-type AMY1 (Juge *et al.*, 1996), forming large haloes on starch agar plates after 12−15 h, this host was chosen for production of the AMY1Δ9 mutant. Indeed, transformants harbouring the plasmid which encodes this truncated gene also gave rise to large haloes on starch plates around colonies after 2 d. *P. pastoris* thus also secreted the AMY1Δ9 mutant in high amounts. AMY1Δ9 retained activity over longer periods of storage at 277 K.

2.2.4. Enzymatic properties of the AMY1Δ9 mutant. The specific activity towards insoluble Blue Starch was essentially the same for AMY1Δ9 and the corresponding full-length recombinant AMY1.

2.3. Crystallization

2.3.1. Crystallization of recombinant full-length and truncated AMY1 forms. AMY1 (full-length) was first crystallized at 292 K employing the hanging-drop vapour-diffusion method using 21% PEG 8000 as precipitant in 0.1 *M* Tris pH 8.0 with a final protein concentration of 2 mg ml^{-1} in the drop. The protein stock solution consisted of 20 m*M* MES pH 6.7 and 100 m*M* $CaCl_2$. As in earlier crystallization trials on this enzyme (Svensson *et al.*, 1987), only two-dimensional crystals were obtained. The size reached after one month was 1 × 0.6 × 0.01 mm. Owing to their morphology, these crystals were mechanically unstable and thus very difficult to handle. It was speculated that the failure to obtain three-dimensional crystals of AMY1 was a consequence of their having a flexible C-terminus compared with the nine-residue shorter C-terminal segment of AMY2. In an attempt to obtain three-dimensional crystals, C-terminally truncated recombinant AMY1 was produced as described above and used for crystallization. Crystals suitable for X-ray diffraction studies were obtained after approximately one month in 20% PEG 8000 using 3% 2-methyl-2,4-pentanediol (MPD) as additive employing Additive Screen 1 (Hampton Research). The initial protein concentration was 5.1 mg ml^{-1} and the protein solution to mother liquor ratio was 2:3. Crystals were grown at 292 K by the hanging-drop vapour-diffusion technique.

Finally, another set of conditions for the C-terminally truncated AMY1Δ9 was found which led to crystals with higher diffraction power and better scaling statistics. The hanging-drop vapour-diffusion method was again used and the drop was prepared by mixing 2 µl of protein solution (5.1 mg ml^{-1}) with 0.5 µl 3%(*v*/*v*) 2-propanol and 2.5 µl well solution containing 500 µl of 20%(*w*/*v*) polyethylene glycol 8000, again resulting from Additive Screen 1. Crystals grew to a typical size of 0.5 × 0.05 × 0.05 mm after three weeks at 290 K.

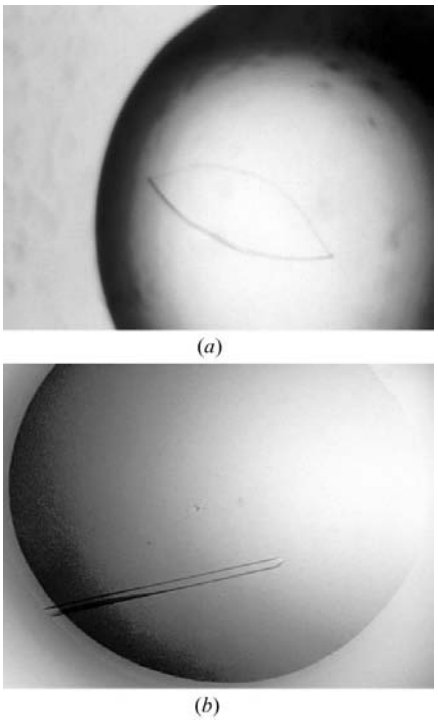

Figure 2
Typical crystals of (*a*) full-length AMY1 and (*b*) C-terminal truncated AMY1Δ9.

2.4. X-ray diffraction and data processing

Diffraction data on crystals of both full-length and truncated AMY1 (first crystallization conditions) were collected on a MAR180 image-plate detector connected to a Rigaku RU-200 rotating-anode generator (Cu $K\alpha$ radiation) with a graphite monochromator. Crystals of full-length and truncated AMY1 diffracted to 2.5 and 2.1 Å, respectively. The integration of the diffracted intensities was achieved with the program *DENZO* (Otwinowski & Minor, 1997) and the data were scaled with the program *SCALA* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994).

Crystals of full-length (Fig. 2) as well as truncated AMY1 (Fig. 2) belong to the orthorhombic space group $P2_12_12$, with unit-cell parameters a = 93.1, b = 74.0, c = 64.5 Å for the truncated form and a = 89.5, b = 73.1, c = 68.1 Å for the full-length form. The space group of the full-length form was only determined/confirmed once the space group of the truncated form was known, since the morphology of full-length AMY1 crystals (Fig. 2) gave rise to a dead zone around one of the twofold axes in the diffraction pattern. The solvent contents of the two crystal forms were 51 and 50%, respectively, with volume-to-mass ratios (Matthews, 1968) of 2.49 and 2.45 Å^3 Da^{-1}. The 1.5 Å resolution diffraction data set was collected under cryo-conditions at 100 K with crystals of type 2 (Fig. 2). Data collection was carried out at the FIP BM30A beamline at the ESRF (European Synchrotron Radiation Facility, Grenoble, France) at a wavelength of 0.9761 Å. The detector used was a MAR345 image-plate system. The crystal was cryoprotected prior to the experiment by rapid soaking in three successive steps in mother liquor containing an increasing concentration of 5, 10 and 15%(*v*/*v*) ethylene glycol cryoprotectant. Integration and reduction of the diffraction data were performed with the same programs as for the two other sets of data. These crystals also belong to the orthorhombic space group $P2_12_12$, with unit-cell parameters a = 88.36, b = 72.82, c = 61.74 Å. Assuming a molecular weight of 44 592 Da (corresponding to truncated AMY1) and one molecule in the asymmetric unit gives a solvent content of 45% and a volume-to-mass ratio, V_M, of 2.23 Å^3 Da^{-1}. Data-collection statistics for the high-resolution data are reported in Table 1.

Table 1
X-ray diffraction data for C-terminally truncated AMY1 (AMY1Δ9).

Values in parentheses are for the highest resolution shell.

No. of observed reflections	271695
No. of unique reflections	63366
Redundancy	4.3 (3.5)
Resolution range (Å)	41.6–1.5 (1.54–1.50)
$I/\sigma(I)$	13.6 (6.2)
Completeness (%)	98.7 (97.3)
R_{sym}† (%)	4.5 (11.9)

† $R_{sym}(I) = \sum |I_n - \langle I \rangle| / \sum I_n$.

2.4.1. Molecular replacement. The molecular-replacement method using the *AMoRe* program (Navaza, 2001), as implemented in the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994), was employed to solve the phase problem. The three-dimensional structure of AMY2 (Kadziola *et al.*, 1994) without the bound calcium ions and water molecules was used as a search model. In a first step, diffraction data from the first crystals of truncated AMY1 in the resolution range 10–4 Å were used in the molecular-replacement search and a unique solution was obtained with a correlation coefficient of 62.1% and an R factor of 35.4%. Once this solution was found, exactly the same procedure was performed with the data from full-length AMY1 and in this case a correlation coefficient of 64.1 and an R factor of 32.3% were found. Nevertheless, the poor-quality data of full-length AMY1 revealed several badly defined regions and in the future the crystal structure of the truncated form will be used as the reference structure for AMY1.

During the refinement of the initially collected data of truncated AMY1, a set of data diffracting to 1.5 Å resolution was collected. Because of the large gain in resolution and badly defined regions in the 2.1 Å resolution structure of truncated AMY1, we decided to continue with the new high-resolution data. These data were used in a molecular-replacement search employing the refined coordinates of the C-terminal truncated data (without calcium ions and water molecules) as a search model,

again using the program *AMoRe* (Navaza, 2001). Diffraction data in the resolution range 15–4 Å were used and a unique solution was obtained with a correlation factor of 62.5 and an *R* factor of 37.8%. With these high-quality synchrotron data from AMY1 it will finally be possible to gain insight into the subtle differences between AMY1 and AMY2.

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