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Studies on the C-terminal region of barley α-amylase 1 with emphasis on raw starch-binding

Brian K. Tibbot, Dominic W. S. Wong* & George H. Robertson

USDA-ARS, Western Regional Research Center, 800 Buchanan Street, Albany, CA 94710 USA; tel.: ++ 1 510 5595877, fax: ++ 1 510 5595777, e-mail: bkt@pw.usda.gov

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The mature form of barley seed, low-pI α-amylase (AMY1) is a polypeptide 414 amino acids in length and possesses a catalytic site and a non-catalytic raw starch-binding (SB) site. In our previous work, a truncated AMY1 cDNA encoding the C-terminal region (134 aa) comprising domain C and part of domain A, but excluding the SB site Trp278-Trp279, was expressed in *Escherichia coli*. The recombinant protein rSB134 bound to cyclohepta-amylose (CHA)-Sepharose and was eluted with free CHA. This work indicated that a second non-catalytic SB site is located in the C-terminal region and binds in the absence of the first SB site and N-terminal region. A shorter truncation rSB62 comprising domain C did not bind, suggesting that domain C alone cannot bind starch and that a portion of domain A is required. In the current study, analysis of a human serum albumin (HSA)-SB fusion rSB68 expressed in the yeast *Pichia pastoris* further suggests that domain C alone cannot bind starch. We also investigated the role of the conserved amino acids VWEK near the C-terminal end of AMY1. Truncated and site-directed mutant forms of rAMY1 expressed in *P. pastoris* had greatly reduced activity, suggesting that the conserved region is required. This region likely plays a role in structural stability.

Key words: amylase, barley, carbohydrate binding, cyclodextrin, Pichia pastoris, raw starch, recombinant protein.

Abbreviations: AMY1, barley seed α-amylase-1, low-pI form; AMY2, barley α-amylase-2, high-pI form; CHA, cyclohepta-amylose; GAMY, *Aspergillus niger* glucoamylase; HSA, human serum albumin; SB, raw starch-binding; SBD, starch-binding domain.

Introduction

α-Amylases (1,4-α-D-glucan-4-glucanohydrolase; EC 3.2.1.1) are endo-glucanases that catalyze the hydrolysis of internal α-1,4-glucosidic linkages in raw and soluble starch, thereby generating smaller dextrins and oligosaccharides. The dextrins generated by α-amylase are hydrolyzed further by exo-glucosidases (e.g. β-amylase, α-glucosidase or glucoamylase) into fermentable sugars, maltose and glucose. Such multi-enzyme processes are vital in nature for germination and early growth of young

* Corresponding author

seedlings and in the malting-brewing, sweetener and bioethanol fuel industries.

α-Amylases constitute a subset of the α-amylase family of enzymes or glycoside hydrolase family 13 (GH-13), which is based on amino-acid sequence similarity (HENRISSAT & BAIROCH, 1993). They are present in animals, plants, fungi, and bacteria. Their sequences, structures, domain organization, evolutionary relationship, and specificities have been reviewed (SVENSSON, 1988; HUANG et al., 1992; JESPERSEN et al., 1993; JANECEK, 1994, 1997; MACGREGOR et al., 2001; PUJADAS & PALAU, 2001). As a group, they have low amino-acid sequence similarity; however five or more short conserved regions are present and comprise the catalytic/substrate binding sites. α-Amylases possess at least three distinct domains, including a common $(\beta/\alpha)_8$ barrel-fold structure comprising domains A and B. Domain A and its protruding loop, domain B located between β_3 and α_3, form the barrel structure and contain the catalytic region. Domain C is situated at the C-terminal end and consists of a β-sheet structure. Little is known about domain C, but it is required for enzymatic activity (HOLM et al., 1990; VIHINEN et al., 1994; JANECEK, 1997).

Several amylolytic enzymes contain a starch-binding domain (SBD). They are found in bacterial and fungal α-amylases (GH-13), bacterial cyclodextrin glucanotransferases (GH-13), bacterial β-amylases (GH-14) and fungal glucoamylases (GH-15). These SBDs are approximately 100 amino acids in length and affixed at the C-terminal end, with the exception of an N-terminal SBD in *Rhizopus oryzae* glucoamylase. In *Aspergillus niger* glucoamylase (GAMY), starch binding is a prerequisite for native starch hydrolysis. The SBD cooperates with the catalytic domain and accelerates hydrolysis in two ways. It increases the local substrate concentration at the active site of the catalytic domain and disrupts starch surface structure (HAYASHIDA et al., 1989; SOUTHALL et al., 1999). Interestingly, the SBDs from these three distinct GH families have significant sequence homology (SVENSSON et al., 1988, 1989; JANECEK & SEVCIK, 1999), in spite of the fact that their catalytic domains have dissimilar sequences and barrel-fold structures. α-Amylase and β-amylase are both $(\beta/\alpha)_8$ barrel proteins but their TIM-barrels are very different. GAMY has an $(\alpha/\alpha)_6$ barrel-fold structure. SBD homology is more correlated with the species origin rather than the given GH family, indicating that this independent structure, which has been classified as carbohydrate-binding module family 20 (CBM20; COUTINHO & HENRISSAT, 1999), was attached or removed from these enzymes during evolution. The SBD from GAMY G1 and domain E from *Bacillus macerans* cyclodextrin CGTase, expressed in *E. coli* as β-galactosidase fusion proteins, retain their SB function (DALMIA et al., 1995). The SBD requirements of these two enzymes differ. GAMY G2, which lacks the SBD but possesses the catalytic domain and *O*-glycosylated S/T-rich linker region (SVENSSON et al., 1986), has reduced activity on raw starch but retains activity on soluble starch (SVENSSON et al., 1982). However CGTase lacking domain E is inactive (VIHINEN et al., 1994); thus this structure is required for basic function. Not all members of the above GH families contain CBM20 and the prominent linker, including plant α-amylases. However, these latter are capable of degrading raw starch and seemingly compensated or progressed during evolution by creating SB sites within their existing structures.

α-Amylases from cereals and other plants have high sequence homology, and their classification and evolution has been reviewed (HUANG et al., 1992). Germinated barley seeds contain two α-amylase isozymes, the low-pI (AMY1) and high-pI (AMY2) forms, which are encoded by separate genes, the expression of which is hormonally regulated (ROGERS & MILLIMAN, 1983; ROGERS, 1985). Barley AMY1 and AMY2 have 80 % amino-acid sequence identity but differ in their physical properties, substrate preferences, and calcium requirements. AMY1 possesses nine extraneous amino acids at the C-terminus, in relation to the conserved AMY2 and other plant α-amylases. As many as seven of these residues are removed by malt carboxypeptidases (SOGAARD et al., 1991), and as many as four residues are cleaved off when expressed in *P. pastoris* (JUGE et al., 1996). AMY1 is more effective than AMY2 in hydrolyzing native starch granules (MACGREGOR & MORGAN, 1986). The crystal structure of AMY2 is known (KADZIOLA et al., 1994). Based on AMY2, AMY1 (Fig. 1A) possesses the domain organization A (V3-N346), B (V90-L153) and C (A353-S414), of which C is a five-stranded anti-parallel β-sheet structure.

In AMY1 and AMY2, starch binding is also a prerequisite for granule hydrolysis, however a starch structure-disruption function has not been reported. A non-catalytic SB site was previously identified in AMY1 and AMY2. This site is located at W276/W277 in AMY2 (W278/W279 in AMY1) and is exposed on the surface of the α_{6b} helix in the $(\beta/\alpha)_8$ structure of domain A. These studies employed chemical modification of native AMY2

A **Barley low-pI alpha-amylase amino acid sequence and organization**

```
   signal peptide        1
mgkngslccfsllllllaglasgHQ<A>VLFQGFNWESWKQSGGWYNMMMGKVDDIAAAGV
THVWLPPPSHSVSNEGYMPPRLYDIDASKYGNAAELKSLIGALHGKGVQAIADI<B>VINHR
CADYKDSRGIYCIFEGGTSDGRLDWGPHMICRDDTKYSDGTANLDTGADFAAAPDIDHL<B>
NDRVQRELKEWLLWLKSDLGFDAWRLDFARGYSPEMAKVYIDGTSPSLAVAEVWDNMATGGD
GKPNYDQDAHRQNLVNWVDKVGGAASAGMVFDFTTKGILNAAVEGELWRLIDPQGKAPGVMG
  281                          303                          331
WWPAKAATFVDNHDTGSTQAMWPFPSDKVMQGYAYILTHPGIPCIFYDHFFNWGFKDQIAAL
          347       353
VAIRKRN<A>GITATS<C>ALKILMHEGDAYVAEIDGKVVVKIGSRYDVGAVIPAGFVTSAH
                    414
GNDYAVWEKNGAAATLQRS*
```

B **PCR synthesis of AMY1 cDNA encoding a 134 aa C-terminal region**

```
                    281                          414
      ThrSerThrGlyGlyAlaLysAlaAlaThrPhe---GlnArgSer*
---------- oligo up ---------------→
CGGGCTACTAGTACTGGGGGCGCCAAGGCCGCCACCTTC---CAACGGAGCTGAAGTCTGC NotI
      SpeI           CGGTTCCGGCGGTGGAAG---GTTGCCTCGACTTCAGACGCCGGCGTAAGAATG
                                      ←-------- oligo down -----------

      GluPheAlaLysAlaAlaThrPhe---GlnArgSer*
   ------- oligo up --------→
5'-CCGGAATTCGCCAAGGCCGCCACCTTC---CAACGGAGCTGAAGTCTGC NotI         -3'
3'-   EcoRI CGGTTCCGGCGGTGGAAG---GTTGCCTCGACTTCAGACGCCGGCGTAAGAATG-5'
                               ←---------- oligo down ---------
```

C **AMY1 fusion proteins for expression in P. pastoris and E. coli**

```
                          | HSA(609aa)|spacer|AMY1 fragment
pPIC-A281/S414 (HSA-SB134) MKWVT---AALGLSTSTGGAKAATF---QRS*
pPIC-G347/S414 (HSA-SB68)  ------------------GITATS---QRS*

                          | N-terminal His-Tag  | adapter |AMY1 fragment
pET-A281/S414 (HT-SB134)  MGSSHHHHHHSSGLVPRGSHMLEKREAEAYVEFAKAATF---QRS*
pET-A353/S414 (HT-SB62)   --------------------------EAYALKILM---QRS*
```

Fig. 1. Construction of AMY1 SB fusion protein expression vectors. (**A**) AMY1 complete amino-acid sequence. The four conserved regions specific for all α-amylases are underlined, as well as W278/W279, a previously identified SB site. Domains A, B and C are depicted, based on AMY2. The first and last residues of the 134 and 62 C-terminal amino acids, A281-S414 and A353-S414, are in bold. (**B**) PCR-amplification of the cDNA region encoding A281-S414. Synthetic oligonucleotides served as upstream and downstream primers (bold), also incorporating restriction sites and stop codon (*) as noted. (**C**) Amino acid sequence of HSA-SB and HisTag-SB fusion proteins. The cDNAs were digested with *Spe*I/*Not*I or *Eco*RI/*Not*I then ligated into expression plasmids for *P. pastoris* pPIC3.5-HSA or *E. coli* pET15, respectively, downstream and in frame with the region encoding N-terminal polypeptides HSA and HisTag. The plasmids pPIC-A281/S414 and pET-A281/S414 were generated. AMY1 amino acids are in bold.

(Gibson & Svensson, 1987) and X-ray crystal structure analysis of an AMY2-acarbose inhibitor complex (Kadziola et al., 1998). Site-directed mutagenesis of rAMY1 expressed in yeast was performed (Sogaard et al., 1993). W279A resulted in a ten-fold reduction in adsorption onto granular starch, whereas production of W278A failed. W278 may be critical for structural stability, in addition to binding, and is conserved in cereal α-amylases.

We recently determined that AMY1 possesses a second non-catalytic SB region. The second site is located in the C-terminal region, downstream of W278/W279. These studies utilized an 11 kDa tryptic fragment A283-X derived from native AMY1 (Wong et al., 2000), and a HisTag-rAMY1 A281-S414 fusion (rSB134) expressed in *E. coli* (Tibbot et al., 2000). Both fragments, although devoid of the AMY1 N-terminal, catalytic and W278/W279 regions, were capable of binding to CHA. Domain C, in the form of HisTag-rAMY1 A353-S414 (rSB62), in itself appears not to be sufficient for binding. Physical independence was emphasized in this experiment and accomplished by expressing the fragments in *E. coli*. However it required the solubilization and denaturation of inclusion bodies with urea, and proper refolding of each construct prior to CHA-affinity chromatography.

Several aspects of AMY1 are still not entirely understood. In follow-up to our previous study, HSA-SB fusions were expressed in the yeast *P. pastoris* where proper folding is more assured. We also elucidated the structure/activity roles of the extraneous and conserved sequences near the C-terminus by truncation and site-directed mutagenesis. Modified forms of rAMY1 were expressed in *P. pastoris* and their activities determined with soluble starch and maltoheptaose as substrates.

Material and methods

Materials

All materials were purchased from the noted vendors. Chemicals: Fisher Scientific (Pittsburgh, PA), Mallinckrodt Inc. (Paris, KY) and Sigma Chemical Co. (St. Louis, MO); microbiological culture medium components and Agar (Difco Laboratories, Detroit MI); standard and low melting gel agarose (BioWhittaker Molecular Applications, Rockland ME); DNA restriction enzymes and agarase (New England Biolabs, Beverly MA); oligo-nucleotides were synthesized and purified by Biosynthesis Inc. (Lewisville, TX) and MWG Biotech Inc. (High Point, NC); P. pastoris methylotrophic strains GS115 (his4, AOX1, AOX2) and GS115 Albumin (HIS4, aox1: HSA, AOX2; Barr et. al, 1992) and shuttle vectors pPIC9 and pPIC3.5, with or without the Saccharomyces cerevisiae N-terminal α-factor secretory peptide, were from Invitrogen Inc. (San Diego, CA); pPIC3.5-HSA was recreated in our laboratory using the above materials and PCR, then modified to pPIC3.5-HSA-Spe for fusion studies. Native barley α-amylase from malt (A-2771) and amylase assay kit (Procedure No. 577) containing 4,6-ethylidine α-D-maltoheptaside-ρ-nitrophenyl (Et-G_7-PNP) were from Sigma.

AMY1 cDNA synthesis, truncation and mutagenesis

A barley (*Hordeum vulgare* L. cv. Himalaya) low-pI α-amylase cDNA clone (AMY1) was isolated in our laboratory (Wong et al., 2002) from an aleurone tissue cDNA library contained in Lambda ZapII (Stratagene, LaJolla CA). DNA sequencing of the 5' and 3' ends indicated that the clone was essentially identical to the AMY1 cDNA clone E first reported (Rogers & Milliman, 1983; GenBank: J01236). However, peptide sequencing of purified native AMY1 (Wong et al., 2000) and rAMY1 (Andersen et al., 1994) identified a Val instead of Ala at position 284 resulting in a greater identity with AMY2. DNA manipulations were performed using standard protocols (Sambrook et al., 1989) or as recommended by the manufacturer. Upstream and downstream oligonucleotide primer pairs, containing EcoRI/NotI or SpeI/NotI restriction sites, were designed to and within the AMY1 cDNA region encoding amino acids H1-S414 of the mature polypeptide and native stop codon (Fig. 1). PCR-amplification employed high-fidelity pfuTurbo^{TM} thermostable DNA polymerase (Stratagene) and the cycling program described previously (Tibbot et al., 2000). All constructs were manufactured, transformed and screened in triplicate to ensure reproducibility.

For raw starch-binding studies, HSA-SB fusions were constructed in pPIC3.5. Truncated AMY1 cDNAs were synthesized using the S414 downstream primer plus various upstream primers (A281, S303, G331 and G347). These internal sites are adjacent to homologous regions containing known catalytic or substrate binding sites (Svensson, 1988; Sogaard et al., 1993, Tibbot et al., 2000).

For C-terminal-end studies, truncated cDNAs were synthesized using the H1 upstream primer in combination with variable downstream primers that incorporated a series of artificial stop codons. Amino-acid or codon substitutions were created by exchanging nucleotide(s) in downstream primers with H1-N405 as template. Its C-terminal end is comparable to AMY2 and was fully functional in yeast.

Pichia expression vector construction

AMY1 DNAs were digested with *Spe*I/*Not*I or *Eco*RI/*Not*I, separated by agarose gel electrophoresis and purified using agarase. The DNAs were ligated into their respective expression plasmids, pPIC3.5-HSA-Spe or pPIC9, and transformed into *E. coli* with selection on LB-ampicillin plates. *E. coli* harboring recombinant plasmids, in triplicate, were grown in LB-Amp and plasmid DNA purified using the QIAprep spin miniprep kit (Qiagen Inc., Valencia CA).

Pichia transformation and selection
Recombinant pPIC plasmids (2 μg) were linearized by digestion with *Bgl*II and transformed into *Pichia* spheroplasts (Sreekrishna et al., 1988; Invitrogen, 1996). The cells were dispensed onto regeneration-agar plates lacking histidine. After 6 d of selective growth at 30 °C, colonies representing histidine autotrophs (His^+) were streaked onto minimal-agar plates containing methanol and grown 5 d. Small colonies with a slow methanol utilization phenotype (Mut^s) indicated integration at the native AOX1 gene site.

a-Amylase activity plate screening
Pichia candidates (His^+Mut^s), three each, were streaked onto YPMS induction-starch plates (1% yeast extract, 2% peptone, 1 mM $CaCl_2$, 0.5% methanol as an inducer, 0.2% soluble potato starch substrate-Sigma S-9765, 50 mM succinate-NaOH, pH 5.5, and 1.5% agar) and grown at 30 °C for 5–8 d. Transformants secreting enzymatically active rAMY1 displayed clear halos, in contrast to a turbid background. The plates were stained with iodine vapor for 2 hr then photographed using a digital camera (Alpha Innotech Corp, San Leandro CA), generating black and white prints.

Expression and secretion of rAMY1 from P. pastoris grown in liquid culture
Transformants were inoculated in 2 mL of MGY medium (1% yeast extract, 2% peptone, 1.34% YNB, 0.4 mg/mL biotin, and 1% glycerol) and grown 20 hr at 30 °C with shaking at 225 rpm. The saturated cultures were dispensed into 500 mL Erlenmeyer flasks containing 200 mL of MGY and grown 20 hrs. Cells were collected by centrifugation at 3,000 × g for 10 min. The supernatant was discarded and the cell pellet resuspended in 200 mL of BMMY induction medium (1% yeast extract, 2% peptone, 1.34% YNB, 1% casamino acids, 0.4 mg/mL biotin, 1 mM $CaCl_2$, 1% methanol, and 50 mM succinate-NaOH, pH 5.5) and dispensed back into the flasks. Growth and induction were carried out for 3 d at 30 °C with shaking at 200 rpm. Cell-free supernatant (200 mL) was obtained by centrifugation at 5,000 × g for 15 min. rAMY1 was secreted into the medium via the *S. cerevisiae* α-factor secretion signal sequence (although the AMY1 native signal sequence is functional in *P. pastoris*) and with a yield of 50 mg/L (Juge et al., 1996).

In raw starch-binding studies, HSA-SB fusion proteins ($\leq$ 40 mg/L culture) in the crude samples were dialyzed against AMY buffer (50 mM succinate-NaOH, pH 5.5, 10 mM NaCl, 5 mM $CaCl_2$) prior to CHA-affinity chromatography and SDS-PAGE analysis.

In C-terminal-end studies, rAMY1 proteins ($\leq$ 2 mg/L culture) in the crude samples were precipitated by adding 100 g of ammonium sulfate, resulting in a ~75% saturated solution, then stored 16 hr at 4 °C. The total protein was pelleted by centrifugation at 15,000 × g for 20 min, re-suspended in 20 mL of AMY buffer and dialyzed. These ten-fold concentrated samples were used for Western blot and enzymatic analysis.

Protein gel electrophoresis and detection
HSA-SB and rAMY1 protein samples (10 μL) were subjected to SDS-PAGE on 9% and 11% Tris-glycine gels (Laemmli, 1970), respectively. Pre-stained protein molecular weight markers, native AMY1 and BSA of pre-determined concentrations served as standards. Proteins were detected by Coomassie staining. Proteins were also transferred to nitrocellulose filters and detected immunologically with a rabbit anti-AMY1 polyclonal antibody and goat anti-rabbit horseradish peroxidase conjugate (Tibbot et al., 2000). The recombinant protein concentrations were determined on the gels and filters, aided by digital scanning and spot densitometry, then the samples equalized by either diluting with the negative control, or concentrating via acetone precipitation (3:1) and resuspending in AMY buffer.

CHA-binding assay of rHSA-SB proteins from P. pastoris
HSA-SB fusion proteins (*P. pastoris*) were loaded onto CHA-Sepharose columns equilibrated in buffer (25 mM succinate-NaOH, pH 5.5, 5 mM NaCl, 0.1 mM $CaCl_2$) at 4 °C. The bound fraction was eluted with buffer containing 10 mM CHA. Native AMY1 served as a standard. Detailed methods on CHA-Sepharose preparation and HisTag-SB (*E. coli*) purification were described earlier (Tibbot et al., 2000). The fractions were subjected to SDS-PAGE.

Enzymatic assays
The rAMY1 sample concentrates derived from liquid cultures were assayed with modified maltoheptaose (Et-G_7-PNP) and soluble potato starch as substrates. Incubations were performed at 30 °C for 0, 2 and 16 hr.

With Et-G_7-PNP as substrate, reactions contained sample (3–16 μL), diluent (13–0 μL), AMY buffer (84 μL), 10 mg/mL acetylated BSA (5 μL), and Sigma amylase reagent containing 1.0 mM Et-G_7-PNP (100 μL). Addition of 0.2 M Na_2CO_3 (800 μL) terminated the reactions and permitted color development. Free PNP was determined at A_{405}.

With soluble starch as substrate, reactions contained sample (10–55 μL), diluent (45–0 μL), AMY buffer (45 μL), 10 mg/mL acetylated BSA (5 μL), and 1% w/v soluble potato starch (Sigma S-2630) in AMY buffer (100 μL). Reducing sugars were detected using a dinitrosalicylic acid reagent (800 μL), heated to 100 °C for 10 min. then measured at A_{547} with maltose as standard (Rick & Stegbauer, 1974).

Results and discussion

Raw starch binding fragment
AMY1 contains an additional non-catalytic SB site. This second SB site requires a portion of domain A and domain C for function. In our previous work, a HisTag-AMY1 A281-S414 fusion protein (rSB134) bound CHA-Sepharose while rSB62 (A353-S414) failed to bind (Tibbot et al., 2000).

A

Barley AMY1	A	V	W	E	K	N	GAAATLQRS*
Barley AMY2	A	V	W	E	K	I	*
	A	V	W	Q	K	I	*
	A	V	-	Q	K	I	*
Wheat	C	V	W	E	K	S	GL*
Rice (Σ^9 consensus)	X(V,I)		W	E	K	X	($X_{10\text{-}19,*}$)
Oat	A	V	W	E	K	S	X_6*
Maize	C	V	W	E	K	H	X_{10}*
Bean (kidney & mung)	A	V	W	E	*		
Potato	A	V	W	E	Q	K	A*
	A	V	-	Q	K	*	
Arabidopsis	A	V	W	E	K	K	*
E. coli	A	H	W	Q	K	I	X_{43}*

B

	% Activity			
	Plate	Broth		1 401 405 414
Truncation	Starch	PNPG7	Starch	! ! ! !
AMY414 (-0)	100	100	100	HQ---AVWEKNGAAATLQRS*
AMY405 (-9)	100	101	103	HQ---AVWEKN*
AMY404 (-10)	100	103	109	HQ---AVWEK*
AMY403 (-11)	10	13	9	HQ---AVWE*
AMY402 (-12)	0	<1	0	HQ---AVW*
AMY401 (-13)	0	<1	0	HQ---AV*

C

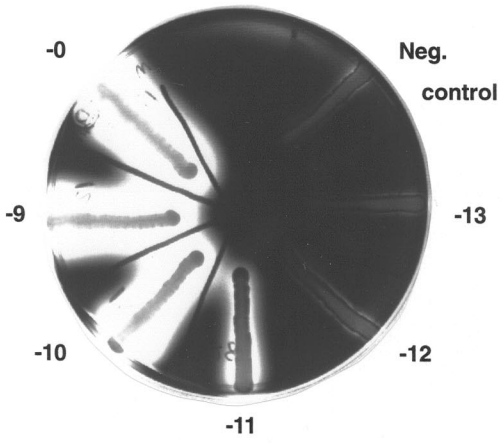

Fig. 2. Truncation of amino acids at the C-terminus of rAMY1 and their effects on activity. (**A**) The C-terminal end of AMY1 versus similar α-amylases. Amino-acid sequence data were acquired from Genbank accessions online (`http://www.ncbi.nlm.nih.gov`). A series of truncations to the 3' end of AMY1 H1-S414 cDNA, representing a loss of 9 to 13 C-terminal amino acids, were synthesized via PCR using various downstream primers. Truncated cDNAs were ligated into pPIC9, a *P. pastoris* secretion expression vector. (**B**) rAMY414 truncations secreted from *Pichia* grown in liquid culture and their activity on maltoheptaose (as Et-G_7-PNP) and soluble starch. Percent activities relative to rAMY414 (100%) and from which 3.2 ρmol of PNP and 1.8 ρmol of reducing sugar were generated per μL of sample concentrate per hr. *Pichia* transformed with pPIC9 served as a negative control. (**C**) Transformants grown on YP agar plates containing methanol and soluble potato starch. After 8 d of growth at 30 °C, the plates were exposed to iodine vapor. Clear halos represent starch hydrolysis while the purple background comprises an iodine-starch complex.

In the current study, AMY1 polypeptides were fused to the C-terminus of HSA (Fig. 1C) and expressed in *P. pastoris*. HSA (68 kDa) and HSA-SB fusions containing G347-S414 (domain C) and G331-S414 (A-α_{8b}, C), 76 and 78 kDa respectively, failed to bind CHA-Sepharose (results not shown). Production of A281-S414 (A-$\beta_7\alpha_7\beta_8\alpha_8$, C), S303-S414 (A-$\alpha_{7b}\beta_8\alpha_8$, C) and longer HSA fusions was unsuccessful. These studies, and the analysis of AMY1 tryptic fragments (Wong et al., 2000), suggest that domain C alone is not sufficient for binding. However three possibilities remain. The second SB site is located within the 72 aa region comprising A281-S352 (A-$\beta_7\alpha_7\beta_8\alpha_8$); this portion of domain A and C are required for an appropriate folding structure; or multiple residues may participate in binding and they may be distributed in both A and C.

Identification of the amino acid(s) contributing to the second SB site would be invaluable. Amino acids D291, W299 and W330 may be of interest. W299 and W330 are conserved in plant α-amylases (Wong et al., 2000), and are located on loops connecting helices α_{7a} with α_{7b} and α_{8a} with α_{8b}, respectively. Although chemical modification studies suggest W299 and W330 do not participate in CHA binding (Gibson & Svensson, 1987), their involvement should not be ruled out. The

A

Mutant	% Activity Plate Starch	Broth PNPG7	Broth Starch	1 !	401 !	405 !	414 !
AMY414	100	117	125	HQ---A	V W E K N		GAAATLQRS*
AMY405	100	100	100	-----A	V W E K N	*	
V401A	10	8	13	-----A	*A* W E K N	*	
V401I	100	54	38	-----A	*I* W E K N	*	
W402A	100	61	48	-----A	V *A* E K N	*	
W402F	100	79	60	-----A	V *F* E K N	*	
E403A	10	17	13	-----A	V W *A* K N	*	
E403D	10	25	42	-----A	V W *D* K N	*	
E403Q	50	58	52	-----A	V W *Q* K N	*	
K404A	100	69	63	-----A	V W E *A* N	*	
K404R	100	64	63	-----A	V W E *R* N	*	

B

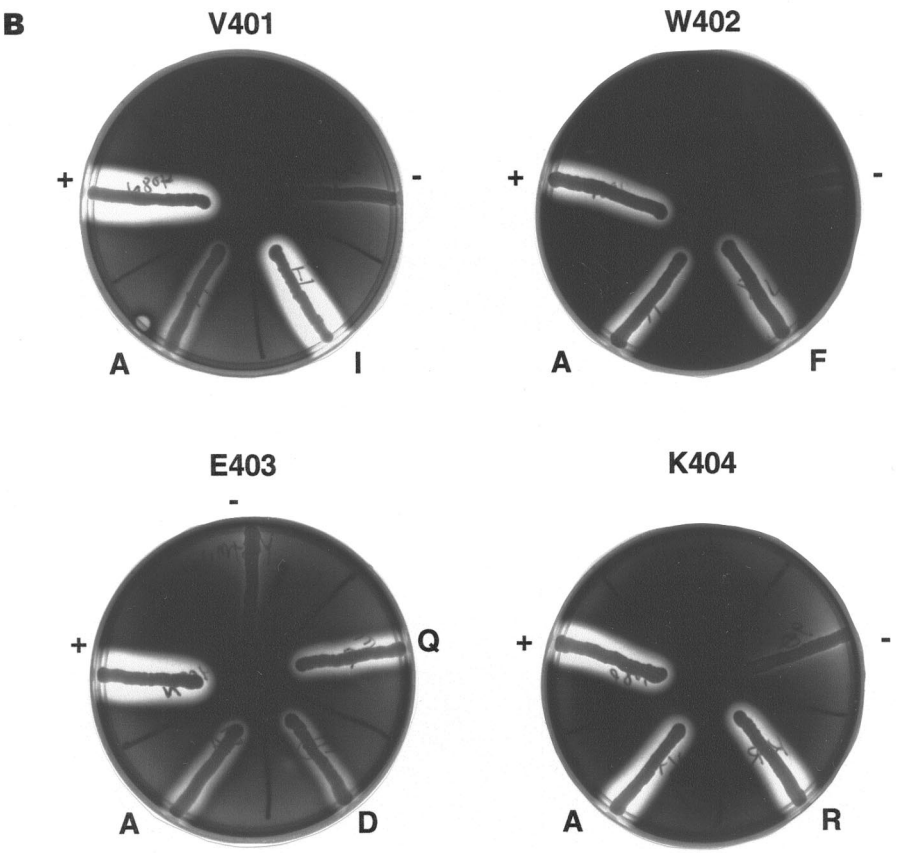

Fig. 3. Mutagenesis of the conserved region near the C-terminus of rAMY1 and effects on activity. AMY1 H1-N405, with a series of amino acid substitutions to the conserved region VWEK, was synthesized via PCR using various downstream primer mutants. (**A**) rAMY405 mutants secreted from *Pichia* grown in liquid culture and their activities on Et-G_7-PNP and soluble starch. Percent activities relative to rAMY405 (100%), from which 3.4 ρmol of PNP and 1.3 ρmol of reducing sugar were generated per μL of sample concentrate per hr. (**B**) Activity from *Pichia* grown on plates containing soluble starch. rAMY405 (+) and pPIC9 (-) served as controls.

binding site in pig pancreatic α-amylase, proposed to be required for hydrolysis of larger substrates, is located on the surface in α_8, as deduced by inhibiting activity via limited proteolysis and antibodies (DESSEAUX et al., 1991). The relative affinities of the two non-catalytic SB sites for CHA and raw starch were not determined in our studies. It is conceivable they have separate roles, but together contribute to enhanced raw-starch binding and degradation.

Truncation of the C-terminus

In our second study, the function of domain C and the C-terminus were investigated. The ten amino acids at the C-terminus of rAMY1, including the nine extraneous residues (Fig. 2A), were not required for enzymatic activity, as determined by the series of truncations expressed in yeast. Yeast containing the truncation rAMY1Δ-10C had full activity (Figs 2B,C). However, rAMY1Δ-11C thru -13C, directed within the conserved region 401VWEK404, had little to no activity. Results were similar with samples derived from the broth of cells grown in liquid culture, with soluble starch and Et-G_7-PNP as substrates (Fig. 2B) versus cells grown on plates containing soluble starch (Fig. 2C). The K404, and preceding amino acids, appear to be essential. Since AMY1 tolerates extraneous sequences, fusion studies at the C-terminus of H1-S414 or H1-N405 may be possible. However fusions positioned at or just upstream of K404 could be problematic.

Mutagenesis of the C-terminus

Amino-acid substitution experiments exploited the region VWEK. As a whole, mutants possessing a conservative substitution, an amino acid of similar charge and structure, were moderately affected (Figs 3A,B). Mutants with dissimilar amino acid substitutions were moderately to severely affected. However all the mutants contained 10% or more activity, including K404A, E403A and W402A. In the truncation experiment, AMY1 lacking these residues had low to no measurable activity. Prior to this study, W402 and E403 were suspected to be essential, due to their highly conserved and possibly reactive nature. However these results suggest that the compositions of these residues do not appear absolutely critical for activity.

The pattern seen in the truncation and mutagenesis experiments, suggests that this region contributes to structural stability and not catalytic or substrate binding. Comparable results were obtained with *Bacillus stearothermophilus* α-amylase (HOLM et al., 1990; VIHINEN et al., 1994), though their C-terminal amino-acid sequences are dissimilar from AMY1. The VWEK region in AMY1 likely interacts with the remaining structure, thereby creating stability. In AMY2, the negatively-charged side chain of E401 is in close proximity to the positively charged R343 and H344, thus creating possible intramolecular salt-bridge(s) linking C-β_5 with A-α_{8b} (KADZIOLA et al., 1994). In AMY1, these positions correspond to E403, R345 and N346, which would give rise to a salt-bridge linking E403 with R345. Coincidentally, this dipeptide region overlaps with AMY1 343RKRN346, a potential yeast KEX2 endoprotease cleavage site, which is not present in AMY2 341RTRH344. The inactive constructs, in general, expressed ΔrAMY1 at a 4–20 -fold lower yield, as estimated from Western blots (data not shown). An enzyme with a compromised structural stability may be synthesized and secreted less efficiently, and be more subject to degradation. Lastly, none of the mutants had significantly greater activity than the wild-type. This region may be nearly optimized during evolution, in context with the other domains.

Future perspectives

Isolation of the AMY1 SB region gives a better understanding of barley α-amylase structure, function, biochemistry, and enzymology. It is a foundation for and encourages future investigations. Inhibition or enhancement of SB function *in vivo* could be useful for biological and malt preparation studies. Experiments to minimize the second SB segment and pinpoint the critical amino acids are in progress. Site-directed mutagenesis of AMY1 and its SB sites and the resulting effect on activity and binding of CHA, raw starch, soluble starch, and smaller substrates will lead to a greater understanding of the enzyme's function. Random mutagenesis coupled with high-throughput screening is in progress. Together, these findings provide the experimental groundwork leading to advanced engineering of an enzyme with enhanced properties.

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