Isozyme hybrids within the protruding third loop domain of the barley α-amylase $(\beta/\alpha)_8$-barrel Implication for BASI sensitivity and substrate affinity

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Received 8 February 1995; revised version received 11 March 1995

Abstract **Barley α-amylase isozymes AMY1 and AMY2 contain three structural domains: a catalytic $(\beta/\alpha)_8$-barrel (domain A) with a protruding loop (domain B; residues 89–152) that binds Ca^{2+}, and a small C-terminal domain. Different parts of domain B secure isozyme specific properties as identified for three AMY1–AMY2 hybrids, obtained by homeologous recombination in yeast, with crossing-over at residues 112, 116, and 144. The AMY1 regions Val^{90}–Thr^{112} and Ala^{145}–Leu^{161} thus confer high affinities for the substrates *p*-nitrophenyl α-D-maltoheptaoside and amylose, respectively. Leu^{117}–Phe^{144}, and to a lesser degree Ala^{145}–Leu^{161}, are critical for the stability at low pH characteristic of AMY1 and for the sensitivity to barley α-amylase/subtilisin inhibitor specific to AMY2.**

Key words: α-Amylase/subtilisin inhibitor; Domain function; Isozyme hybrid; Recombinant α-amylase; Yeast homeologous recombination; Barley

1. Introduction

α-Amylase (α-1,4-D-glucan glucanohydrolase, EC 3.2.1.1) catalyses the hydrolysis of internal α-1,4-glucosidic bonds in starch and related oligo- and polysaccharides. The three-dimensional structures of the enzymes from *Aspergillus oryzae* (Taka-amylase A) [1], porcine pancreas (PPA isoform I) [2], *Aspergillus niger* (acid α-amylase) [3], and barley malt (isoform AMY2–2) [4], are organised in three domains. AMY2–2 thus contains an N-terminal catalytic $(\beta/\alpha)_8$-barrel fold (domain A, residues 1–88 and 153–350) with a $\beta\rightarrow\alpha$ loop (domain B, residues 89–152) protruding at the third β-strand, and a C-terminal five-stranded β-sheet (domain C, residues 351–403) [4]. The functional roles of six invariant residues located at and near the catalytic site have been examined by mutational analysis ([5]; see [6] for a review). Little is known, however, about domain B which, despite its key role in binding of structural Ca^{2+} [3,4] and contribution to substrate binding [1,7], is highly variable in the actual $(\beta/\alpha)_8$ protein family that includes α-amylases, cyclodextrin glucanotransferases, and most other amylolytic enzymes [6,8–10].

Germinating barley seeds produce two α-amylase isozyme families distinguished by low (AMY1) and high pI (AMY2) and having 80% sequence identity [11–13]. Compared to AMY2, AMY1 binds Ca^{2+} firmly, is considerably more stable at low pH, degrades starch granules and oligosaccharides at higher rates, and shows highest substrate affinity in general; AMY2 in contrast is most active on amylose and less heat sensitive [11,14–18]. Finally, the α-amylase/subtilisin inhibitor BASI from barley seeds only inhibits AMY2 [19,20]. AMY1 and AMY2 are thus attractive candidates for investigations in structure/function and structure/stability relationships of α-amylases.

Heterologous expression of AMY1 and AMY2 [21] in conjunction with in vivo homeologous recombination in yeast, efficient in producing cDNA inserts encoding bipartite isozyme hybrids [22,23], enables correlation of particular structural regions with specific enzymic or physico-chemical properties. The first 54 residues of AMY1, but not of AMY2, ensure proper folding of the enzyme and secretion from yeast [24]. Furthermore residues 90–160 (in AMY2; 91–161 in AMY1) that essentially comprise domain B [4] are associated with several isozyme distinct properties, of which the sensitivity of AMY2 to BASI is the most remarkable [18]. In the present study, the three different hybrids that cross-over from AMY1 to AMY2 at residues 112, 116, and 144, respectively, dissect parts of domain B important for recognition of BASI, high substrate affinity, and stability at low pH.

2. Materials and methods

2.1. Materials

p-Nitrophenyl α-D-maltoheptaoside and yeast α-glucosidase were purchased from Boehringer; DP17 (degree of polymerization 17) amylose EX-I was from Hayashibara Chemical Laboratories; insoluble blue starch from Pharmacia; and maltose from Sigma. Acarbose was a gift of Dr. E. Möller (Bayer AG, Wuppertal). Restriction enzymes (*Nar*I, *Nhe*I, *Xma*III) from Boehringer were used according to the manufacturer's recommendation. Barley and malt were supplied by Carlsberg Maltings. AMY2 from malt (cvs. Triumph or Menuet) [16,25], recombinant AMY1 from yeast [21,26], and BASI from barley seeds (cv. Piggy) [27] were purified as reported previously.

2.2. Strains, plasmids, hybrid cDNAs, fermentation and α-amylase purification

Escherichia coli DH5 (*supE*44, hsdR17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1) was used for plasmid isolation and *Sacharomyces cerevisiae*

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Abbreviations: AMY1 and AMY2, barley α-amylase isozymes 1 and 2; AMY1-(1–112)–AMY2-(112–403), etc., hybrid containing His^1–Thr^{112} of AMY1 and Pro^{112}–Ile^{403} of AMY2, etc.; BASI, barley α-amylase/subtilisin inhibitor; pNPG7, *p*-nitrophenyl α-D-maltoheptaoside.

SSDI 0014-5793(95)00291-X

S150-2B (*Mata*, *leu2*-3, *leu2*-112, *his3*-D, *trp1*-289, *ura3*-52) for expression of *AMY1-AMY2* hybrid cDNAs. The parent plasmids ΔP7 and pBAH15 encoding AMY1 and AMY2, respectively, and selected AMY1–AMY2 hybrid plasmids are from house collections [18,24]. A hybrid cDNA library was obtained by co-transformation of yeast with linearized gapped (*Nar*I–*Nhe*I) ΔP7 and an overlapping double-stranded *Xma*III restriction fragment of pBAH15 [18,24]. Standard techniques were used for DNA manipulation [28] and sequencing [29]. Yeast was transformed by the spheroplast method [30]. Transformed yeast harboring a hybrid gene was grown in a 10 L MBR BioReactor for 4 days at 25°C and cells were separated by centrifugation at 4°C. α-Amylase was purified from the supernatant on β-cyclodextrin-Sepharose [26] and checked by SDS-PAGE and by IEF, and stained for protein and activity towards soluble starch (zymogram) [18,26].

2.3. Analyses

N-Terminal sequencing (0.1 nmol protein) was performed using an Applied Biosystems Model 470A sequencer equipped with Model 120A PTH analyzer. Amino acid contents (0.2–1.0 nmol protein hydrolysed in 6 M HCl, 110°C, 24 h) were determined using a Pharmacia/LKB Alpha Plus amino acid analyzer. The concentration of α-amylase or BASI was determined spectrophotometrically at 280 nm using $A_{1\,cm}^{1\%} = 24$ [25] and $A_{1\,cm}^{1\%} = 13$ [27], respectively, or by amino acid analysis.

2.4. α-Amylase activity

Hydrolysis of pNPG7 by α-amylase (1–5 nM) at pH 6.7 (20 mM MES, 1 mM $CaCl_2$) was followed at 30°C at 11 [pNPG7] (0.1–20 mM) in a coupled assay using yeast α-glucosidase (25 U/ml) [5]. Hydrolysis of amylose DP17 by α-amylase (1–5 nM) at pH 5.5 (20 mM sodium acetate, 5 mM $CaCl_2$, 0.5 mg/ml bovine serum albumin) at 37°C was measured at 7 [amylose] (0.06–10.0 mg/ml) by reducing sugars analyses using the copper-bicinchonitate method on aliquots removed at suitable time intervals [5]. The α-amylase (1–2 nM) hydrolysis of insoluble blue starch (6.25 mg/ml, 4 ml) at 37°C in the above buffer was stopped after 15 min by addition of 0.5 M NaOH (1 ml), followed by centrifugation and the absorbance of the supernatant was measured at 620 nm [5]; kinetic parameters were determined using 13 [insoluble blue starch] (0.4–10 mg/ml). 1 U is defined as the amount of α-amylase that gives an increase in $1 \cdot 10^{-3}$ in A_{620}/ml over 15 min in a final volume of 5 ml. α-Amylase (30 nM) was preincubated with the inhibitor acarbose (0.5–50 μM) for 15 min at pH 5.5 (20 mM sodium acetate, 5 mM $CaCl_2$, 0.5 mg/ml bovine serum albumin) at 25°C, the substrate amylose (6.25 mg/ml) was added at the given [acarbose] and activity measured as above. $K_{i,app}$ was calculated as $(A_x - A_0) \times L_x/(A_{max} - A_x)$, where A_{max} and A_x are A_{540} values at 0 μM and other [acarbose], L_x, respectively; A_0 is the blank. Kinetic parameters, K_m and k_{cat} were calculated from initial velocities using the Erithacus-software GraFit (version 3.0). The inhibition of α-amylase (0.3 μM) by BASI was determined from the residual activity towards insoluble blue starch after 15 min preincubation at 24 [BASI] (3 nM–30 μM) at pH 8 (40 mM Tris-HCl, 5 mM $CaCl_2$, 0.5 mg/ml bovine serum albumin) and 37°C. The extent of inhibition by BASI was calculated as $100 \times [1 - (Act_i/Act_0)]$ where Act_i and Act_0 represent the α-amylase activity for mixtures incubated in the presence and the absence of BASI, respectively. K_i for BASI was calculated as described previously [18,27].

2.5. Stability at low pH

α-Amylase (4 nM) was incubated at pH 3.5 (20 mM sodium citrate, 1 mM $CaCl_2$) and 37°C. The residual activity was followed towards insoluble blue starch at varying time intervals.

2.6. Effect of $CaCl_2$

The activity of α-amylase (2 nM) towards insoluble blue starch at varying [$CaCl_2$] (0–100 mM) was determined essentially as described in section 2.4 but in the presence of 0.05 mg/ml bovine serum albumin.

3. Results and discussion

We found recently that the structure of domain B determines distinct properties of AMY1 and AMY2 [18]. The present study focuses on AMY1–AMY2 chimers with junction within domain B (Fig. 1) allowing definition of segments of this domain important for substrate specificity, stability at low pH, and sensitivity to BASI.

3.1. Selection of AMY1–AMY2 hybrid genes

Based on the chimer structure, determined by restriction enzyme analysis and cDNA sequencing, and on a high secretion efficiency in yeast, evaluated by starch-halo-plate assay and Western blot analysis [24], three clones were selected from our hybrid cDNA library (see section 2.2). The recombination events had occurred at nucleotides 408, 420, or 504 relative to the translational start, which resulted in genes encoding residues 1–112, –116, and –144 of AMY1 and the complementary C-terminal regions of AMY2.

3.2. Purification and structural characterization of barley α-amylase isozyme hybrids

The hybrids AMY1-(1–112)–AMY2-(112–403), AMY1-(1–116)–AMY2-(116–403), and AMY1-(1–144)–AMY2-(144–403) were purified from the culture supernatants by affinity chromatography (see section 2.2) in yields of 0.1–0.2 mg/l and thus retained the capacity of the parents to bind β-cyclodextrin at the surface site [31,32] containing Trp^{276}–Trp^{277} [5,33]. Their N-terminal sequence was found to be His-Gln-Val-Leu-Phe, indicating correct leader peptide processing [21,32,34], and the amino acid compositions were in excellent agreement with the deduced hybrid sequences (data not shown). The molecular weights (SDS-PAGE) were approx. 45 kDa as with the parent

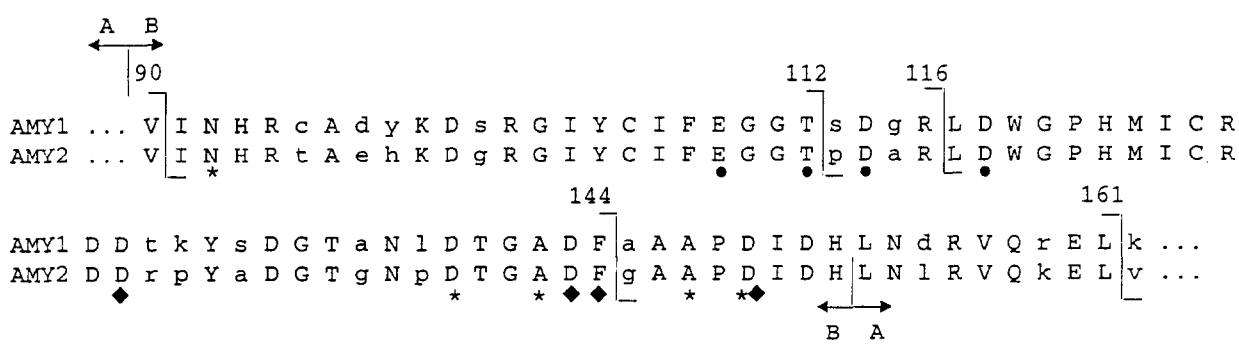

Fig. 1. AMY1 (residues 90–162; [12]) and AMY2 (residues 89–161; [13]) sequence comparison. Numbering starts at the N-terminus of the AMY1 mature isozyme [34]. Conserved residues are in upper case. ⌝⌞ indicates a crossing-over, Ca^{2+} ligands are indicated by * (Ca 500), ● (Ca 501), and ◆ (Ca 502) [4]. The domain B constitutes the third $\beta \rightarrow \alpha$ connecting segment inserted in the $(\beta/\alpha)_8$ barrel domain A between residues 89 and 152 [4].

enzymes, thus over-glycosylation had not taken place. A highly active form of pI 5.3 was seen in the IEF zymogram [26] for all three hybrids (data not shown). AMY1-(1–144)–AMY2-(144–403) contained a minor form of pI 5.0 which was inactive in the zymogram and thus presumably glutathionylated at Cys^{95} [26,34]. The higher pI of 5.5, reported earlier for AMY1-(1–90)–AMY2-(90–403) [18], most likely is explained by the presence of His^{98}, particular to AMY2 (Fig. 1).

3.3. Enzyme–substrate interactions

Depending on the substrate used, pNPG7 or amylose DP17, the hybrids presented different levels of affinity relative to the parent isozymes. The K_m of 0.5–0.7 mM of AMY1-(1–112)–AMY2-(112–403), AMY1-(1–116)–AMY2-(116–403), and AMY1-(1–144)–AMY2-(144–403) (Table 1) for the oligosaccharide pNPG7 is essentially the same as for AMY1-(1–161)–AMY2-(161–403) and hence AMY1-like [18]. Since AMY1-(1–90)–AMY2-(90–403) has a K_m 5 fold higher and thus identical to that of AMY2 [18], we conclude that Val^{90}–Thr^{112} in AMY1 is responsible for the high pNPG7 affinity. Cys^{95}, Asp^{97}, Tyr^{98}, and Ser^{101} are particular to AMY1 (Fig. 1) and may either individually or in concert confer the high substrate affinity. This part of domain B most likely adopts different isozyme specific conformations. AMY1 isolated from malt has Cys^{95} in a disulfide bridge [26,34], while recombinant AMY1 has Cys^{95} either free or glutathionylated being fully and 3% active, respectively. Asp^{97} corresponds to Glu^{96} in AMY2 (Fig. 1) which is salt-bridged to Arg^{55} in the crystal structure [4]. Finally, although AMY1 $His^{93} \rightarrow Asn$ has an increased K_m of 2.1 mM, it also has, in contrast to the hybrids, greatly reduced k_{cat} [5]. The His^{93} is invariant in the family of amylolytic enzymes [6] and plays a crucial role in transition-state stabilization [5]. In the structure [4] Val^{90}–Thr^{112} furthermore is seen to belong to the loop that binds Ca 501 in AMY2, involved perhaps in the specific stimulation of AMY2 activity at high $[Ca^{2+}]$ [18].

The hybrids show a different pattern in the kinetic parameters for hydrolysis of amylose, that covers all ten subsites in the active site of barley α-amylase [16,17], than in pNPG7 (Table 1). AMY1-(1–116)–AMY2-(116–403) and AMY1-(1–144)–AMY2-(144–403), as found for AMY1-(1–90)–AMY2-(90–403) [18], have a K_m similar to AMY2, but the $K_{i,app}$ for inhibition by acarbose, a pseudo tetrasaccharide acting as a transition-state analogue [5], is higher than for AMY2 (Table 1). Apparently the effect of small adverse structural changes probed by acarbose at subsites adjacent to the catalytic site is

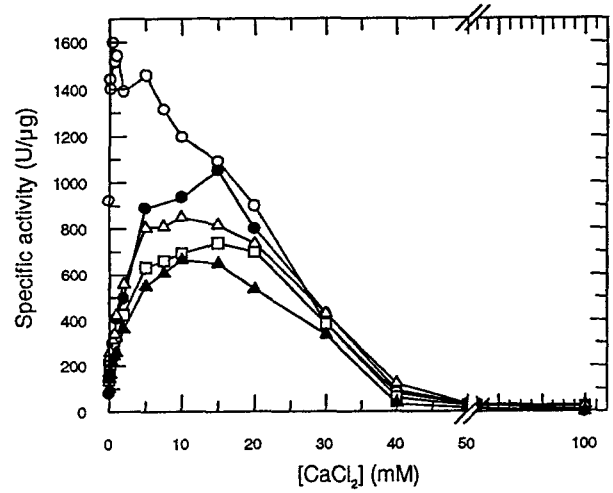

Fig. 2. Influence of $[Ca^{2+}]$ on activity of barley α-amylase isozyme hybrids. AMY2, (●); AMY1-(1–112)–AMY2-(112–403), (□); AMY1-(1–116)–AMY2-(116–403), (△); AMY1-(1–144)–AMY2-(144–403), (▲); AMY1, (○).

diminished with amylose that occupies the long substrate binding crevice. The three intra-domain B hybrids are excellent catalysts having a slightly elevated or AMY2-like k_{cat} for hydrolysis of amylose (Table 1). Since the K_m and $K_{i,app}$ of AMY1-(1–161)–AMY2-(161–403) approach values characteristic of AMY1 (Table 1), residues in Ala^{145}–Leu^{161} of AMY1 apparently confer the higher affinity. Remarkably, AMY1-(1–112)–AMY2-(112–403), however, binds amylose and acarbose more strongly compared to the other two intra-domain B hybrids, the K_m and $K_{i,app}$ being intermediary to values obtained for AMY1 and AMY2. Together these findings suggest that the residues responsible for AMY1-level affinity are located primarily at the beginning (residues 90–112) and at the end (residues 145–161) – two parts of domain B which are seen to interact in the crystal structure [4] – while Ser^{113}–Phe^{144} maintained the AMY2 level of affinity. Clearly subtle variations of the primary structure in domain B modulate the specificity, k_{cat}/K_m being 17–65% higher towards amylose for the four AMY1–AMY2 hybrids crossing-over in the region Gly^{112}–Leu^{161} than for the parent isozymes (Table 1). The pH optima, moreover, gradually shifted to slightly lower values with increasing size of the AMY1 portion (data not shown).

Table 1
Enzymatic properties of parent and hybrid isozymes of barley α-amylase

Enzyme	Substrate						Inhibitor	
	pNPG7			Amylose DP17			Acarbose	BASI
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	K_m ($mg \cdot ml^{-1}$)	k_{cat} (s^{-1})	k_{cat}/K_m ($mg^{-1} \cdot ml\ s^{-1}$)	$K_{i,app}$ (μM)	K_i (nM)
AMY1[a]	0.5 ± 0.1	660 ± 13	1320	0.4 ± 0.1	236 ± 25	590	2.2 ± 1.0	≥ 10^6
AMY1-(1-161)-AMY2-(161-403)[a]	0.5 ± 0.1	327 ± 7	654	0.8 ± 0.2	535 ± 50	669	5.2 ± 2.9	≥ 10^6
AMY1-(1-144)-AMY2-(144-403)[b]	0.7 ± 0.1	352 ± 8	503	1.1 ± 0.3	765 ± 33	695	8.9 ± 3.1	$5 \cdot 10^3$
AMY1-(1-116)-AMY2-(116-403)[b]	0.5 ± 0.1	215 ± 5	448	1.3 ± 0.5	880 ± 50	677	11.5 ± 2.1	0.62
AMY1-(1-112)-AMY2-(112-403)[b]	0.5 ± 0.1	310 ± 5	620	0.7 ± 0.1	659 ± 29	943	4.0 ± 2.8	0.73
AMY1-(1-90)-AMY2-(90-403)[a]	2.4 ± 0.1	400 ± 7	167	1.1 ± 0.2	647 ± 28	539	11.4 ± 4.1	0.33
AMY2[a]	2.5 ± 0.3	405 ± 11	160	1.2 ± 0.1	684 ± 23	570	6.8 ± 2.7	0.22

[a]From [18].
[b]This work.

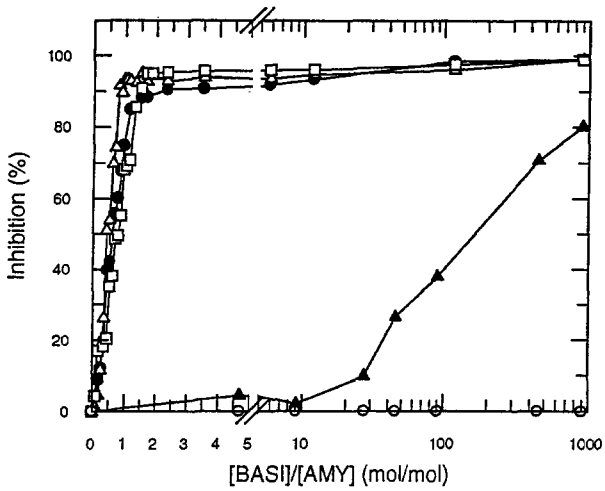

Fig. 3. Inhibition by BASI of barley α-amylase isozyme hybrids. For key to symbols see Fig. 2.

For insoluble blue starch, K_m of the three hybrids was 0.41–0.48 mg/ml, which is similar to K_m for AMY1 and AMY2 of 0.42 and 0.46 mg/ml, respectively.

The k_{cat} values of the chimeric enzymes on the three different substrates: pNPG7 and amylose (Table 1), and insoluble blue starch (Fig. 2) are closest to those of AMY2 (Table 1 and Fig. 2). This may be due to the fact that Asp^{179}, Glu^{204}, and Asp^{289}, which directly participate in catalysis [5,6], belong to the part of domain A which in the hybrids originates from AMY2.

3.4. *Distinct overlapping regions of domain B confer sensitivity to BASI and stability at low pH*

Inhibition by BASI and lack of stability at low pH are characteristics of AMY2 that recur for the earlier described AMY1-(1–90)–AMY2-(90–403) [18]. This behaviour is also shown by AMY1-(1–112)–AMY2-(112–403) and AMY1-(1–116)–AMY2-(116–403) (Figs. 3 and 4), whereas AMY1-(1–144)–AMY2-(144–403) resembles AMY1 (Figs. 3 and 4) and AMY1-(1–161)–AMY2-(161–403) [18]. The AMY2 regions Leu^{116}–Phe^{143} and, with a very minor contribution, Gly^{144}–Leu^{160} thus control the sensitivity to BASI, and the corresponding parts from AMY1 confer significant stability at low pH.

The inhibition constant, K_i, is 10^4 fold higher for BASI inhibition of AMY1-(1–144)–AMY2-(144–403) than of AMY1-(1–116)–AMY2-(116–403), being almost as sensitive as AMY2 having a K_i of 0.22 nM (Table 1). For this part of domain B (Leu^{116}–Phe^{143} in AMY2) that has only a few interactions with other parts [4], five amino acid residues differ between the two hybrids. Among these, two represent conservative substitutions and two are Pro in AMY2 (Fig. 1). AMY2–BASI was recently crystallised [35] and in a preliminary model Arg^{128} of AMY2 (Thr^{129} in AMY1) is seen to interact with BASI (F. Vallée, A. Kadziola, Y. Bourne, B. Svensson and R. Haser, unpublished). We suspect that a salt-bridge to BASI involving Arg^{128} in conjunction with a favourable local conformation of AMY2 secures the sensitivity to BASI. Since BASI is a very modest inhibitor of AMY1-(1–144)–AMY2-(144–403) with a K_i around $5 \cdot 10^3$ nM compared to a $K_i \geq 10^6$ nM for both AMY1-(1–161)–AMY2-(161–403) and AMY1 (Table 1), Gly^{144}–Leu^{160} in AMY2 is not essential in BASI complex formation.

AMY1 is quite stable at pH 3.5 [15,18] and while AMY1-(1–161)–AMY2-(161–403) retained 65% [18] and AMY1-(1–144)–AMY2-(144–403) 30% activity of AMY1 after 5 min at pH 3.5, AMY1-(1–112)–AMY2-(112–403), AMY1-(1–116)–AMY2-(116–403) (Fig. 4), AMY1-(1–90)–AMY2-(90–403), and AMY2 [18] all lost activity completely. It is known that interactions between charged residues can play an important role in protein stability at acidic pH. In the sequence Arg^{116}–Leu^{161} (Fig. 1), a potential candidate for salt-bridge formation is Asp^{154} (Leu^{153} in AMY2) located at the hinge between the domain B and the $(\beta/\alpha)_8$-barrel [4]. Another possible determinant in the acid stability is the short sequence Thr^{129}–Ser^{132} (Arg^{128}–Ala^{131} in AMY2) which is likely to adopt isozyme-specific conformations especially because AMY2 has a non-conserved Cis–Pro^{129} [4].

The three Ca^{2+} in AMY2 bind to domain B (Fig. 1) and presumably enhance its conformational stability [4]. There is no evidence of whether AMY1 contains all three Ca^{2+}, but since the protein ligands are conserved (Fig. 1), it is surprising that the intra-domain B hybrids resemble AMY2 and not AMY1 in that high [Ca^{2+}] is required for maximum activity (Fig. 2). Previously also AMY1-(1–90)–AMY2-(90–403) and AMY1-(1–161)–AMY2-(161–403) were demonstrated to be most active at 5–10 mM Ca^{2+} [18]. Since, compared to AMY2 [4], the structure near Ca 501 (Fig. 1) is most likely perturbed in AMY1-(1–112)–AMY2-(112–403) and AMY1-(1–116)–AMY2-(116–403) and near Ca 502 in AMY1-(1–144)–AMY2-(144–403), respectively, features of AMY1 after residue 161 are probably responsible for the high Ca^{2+} affinity or the lack of requirement for extra Ca^{2+} for optimal activity of this isozyme. Domain B apparently does not fully control the influence of Ca^{2+} on activity.

In conclusion, generation of AMY1–AMY2 isozyme hybrids by in vivo homeologous recombination is an excellent dissecting strategy to establish a functional map of barley α-amylase domain B. In AMY2 three Ca^{2+} contribute to the structural integrity of domain B together with salt-bridges and interactions with the second $\beta \rightarrow \alpha$ connecting loop [4]. As demonstrated in the present work, this particular architecture allows engineering of α-amylase properties by using an isozyme hybrid approach. For example, the hybrid AMY1-(1–112)–AMY2-

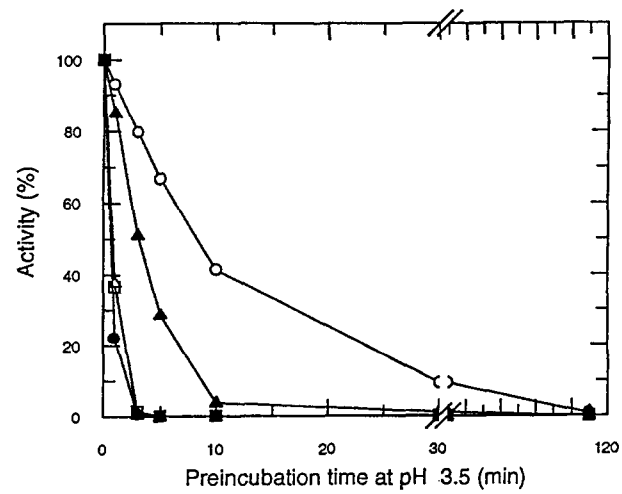

Fig. 4. Stability of barley α-amylase isozyme hybrids at pH 3.5. For key to symbols see Fig. 2.

(112–403) has activity on amylose clearly superior to both parents (Table 1, k_{cat}/K_m values with amylose DP17). This and other intra-domain B hybrids moreover are more active than AMY2 on an oligosaccharide substrate (Table 1, k_{cat}/K_m values with pNPG7). Such improvements in combination with the BASI mediated activity control will be further explored by site-directed mutagenesis of selected residues evidenced by the isozyme hybrids. Ultimately transgenic barley producing engineered α-amylase with enhanced activity can be used for improvement of malt quality [36].

Acknowledgements: The expert technical assistance of Annette Gajhede with enzyme assays and Bodil Corneliussen, Lone Sørensen and Ib Svendsen with amino acid and N-terminal sequence analyses is gratefully acknowledged. Morten Meldgård and Liselotte Møller are thanked for their help with fermentation. N.J. held a short-term postdoctoral EMBO fellowship.

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