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α-Amylases: structure and function

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The present review describes the modular architecture of amylolytic enzymes with different specificity, as seen in crystal structures and given by predicted structures, respectively. Based on known enzyme–substrate interactions in α-amylases, structural features of the catalytic $(\beta/\alpha)_8$-barrel domain can be proposed, which play a role in defining the individual specificities. A yeast expression system has been developed for genes encoding barley α-amylase isozymes. Genetic changes were made to overcome post-translational glutathionylation and C-terminal degradation. Subsequently, three carboxylic acid and two histidine residues from the active site and two tryptophans located in a separate starch-granule binding site, characteristic of cereal α-amylases, were subjected to mutational analysis. The carboxyl groups were all essential for activity, while the histidines were important for transition-state stabilisation. Replacement at the surface site reduced the affinity for starch granules. Finally, properties distinguishing the two isozyme families of barley α-amylase, such as the Ca^{2+}-effect on activity and the sensitivity to the endogeneous inhibitor, BASI, were discussed.

INTRODUCTION

Starch is an important source of energy for animals, microorganisms, and higher plants. Its utilization requires degradation to maltooligodextrins and glucose which is done in nature by a palette of amylolytic enzymes of varying specificity. This paper addresses first the structure and function of amylolytic enzymes in a broad sense with emphasis on characteristic similarities and differences between these enzymes. Then, following a short general introduction to α-amylases, we shall focus on our recent structure/function relationship investigations in the low and high pI barley malt α-amylase isozymes, AMY1 and AMY2. Finally, properties of the barley α-amylase/subtilisin inhibitor (BASI) and its specific interaction with AMY2 will be briefly discussed.

ARCHITECTURE OF AMYLOLYTIC ENZYMES

Classification of enzymes degrading starch and related poly- and oligosaccharides can be made by the following distinctions in their behaviour: (1) endo- versus exo-mode of attack, (2) inversion versus retention of the anomeric configuration of the substrate, (3) preference for poly-, oligo-, or disaccharides, (4) α-(1→4), α-(1→6), or dual bond-type specificity, and (5) hydrolytic versus glucosyl-transfer activity (for a review see Svensson, 1991). By combination of these five categories, one can cover the diversity of reactions catalysed by the starch-degrading and related enzymes. Interestingly, the difference in specificity seems for a large number of these enzymes to be brought about by structural variation of substrate interacting regions of a common polypeptide chain fold motif, the α/β-barrel (MacGregor & Svensson, 1989; Jespersen *et al.*, 1993). Thanks to cloning and sequencing techniques, more than 100 primary structures of amylolytic and related enzymes representing 16 substrate specificities are known today. However, the tertiary structure has been determined by crystallography only for very few of these enzymes: Taka-amylase A (Matsuura *et al.*, 1984); porcine pancreatic α-amylase (Buisson *et al.*, 1987); *Aspergillus niger* α-amylase (Boel *et al.*, 1990); two cyclodextrin glucanotransferases from *Bacillus stearothermophilus* (Kubota *et al.*, 1990) and *Bacillus circulans* (Klein & Schulz, 1991), respectively; soybean β-amylase (Mikami *et al.*, 1990); and very recently barley malt high pI α-amylase, AMY2 (Kadziola *et al.*, 1992) and

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glucoamylase from *Aspergillus awamori* var. X-100 (Aleshin *et al.*, 1992). The α-amylases and CGTases contain the same α/β-barrel fold pattern of the catalytic domain, while the β-amylase adopts a different α/β-barrel fold, and the catalytic domain of glucoamylase belongs to a completely different structural family described as an α/α-barrel, a new folding pattern reported first for the *Clostridium thermocellum* endoglucanase CelD (Juy *et al.*, 1992). The lack of available three-dimensional structures for the vast majority of amylolytic enzymes made us and others consider the application of structure prediction procedures in combination with the known crystal structures for members of the amylase superfamily as a way to exploit the many primary structures (MacGregor, 1988; MacGregor & Svensson, 1989; Raimbaud *et al.*, 1989; Jespersen *et al.*, 1991, 1993). First hydrophobic cluster analysis (Lemesle-Varloot *et al.*, 1990) in conjunction with sequence alignment helped tentative identification of separate domains along the polypeptide chain and secondary structural elements of the α/β-barrel, respectively (Jespersen *et al.*, 1991). This approach provided strong support for the α-amylase fold type being adapted by (1) two exo-α-amylases: maltogenic α-amylase and maltotetraohydrolase; (2) isoamylase, pullulanase, neopullulanase, α-amylase-pullulanase, branching enzyme, oligo-1,6-glucosidase, and a dextranase, all acting on α-1,6-linkages; and (3) a maltase and a maltopentaose-producing α-amylase (Jespersen *et al.*, 1991, 1993). This has major implications for identification of putative functionally important residues and structural motifs. At present no other starch-degrading or related enzyme has been shown to share the fold of either β-amylase or glucoamylase. A less extensive structural similarity has been detected, however, that encompasses short active site regions (Svensson, 1988) containing either an affinity labelled carboxylic acid residue as in sucrase-isomaltase (Quaroni & Semenza, 1976; Hunziker *et al.*, 1986), *Aspergillus* α-glucosidase (Chiba *et al.*, 1990), and dextransucrase (Mooser *et al.*, 1991), or one (Asp 206 in Taka-amylase A) of three invariant carboxylic acid residues in the different enzymes proposed to have a catalytic domain of the α-amylase type. This makes us speculate whether a link exists in the form of a small conformational motif between these other functionally related enzymes and the members of the α-amylase structural family. It further leads to the suggestion that the specific catalytic role (Sinnott, 1990) of these presumed equivalent carboxylic acid residues is to act as enzyme nucleophile (Svensson & Søgaard, 1992). α-Glucosidases with different bond-type and substrate chain length preference may thus either belong to the α-amylase structural family as seen for a maltase from *Saccharomyces cerevisiae* (MacGregor & Svennson, 1989) or to another more distant, if at all structurally related, group of enzymes as discussed above. Several physiologically important enzymes are found in the latter group, including the human lysosomal acid α-glucosidase (Hermans *et al.*, 1991) and different glycosidases from the intestinal brush border. It remains an open question what type of folding pattern these enzymes have. Their long polypeptide chains, however, point to a multidomain architecture as found for the vast majority of starch hydrolases.

The enzymes degrading starch and related poly- and oligosaccharides are built in a modular fashion containing a varying number of structural domains (Jespersen *et al.*, 1991) from one as in soybean β-amylase; two in *Aspergillus* glucoamylase and *Clostridium thermosulfurogenes* β-amylase; three in most α-amylases, yeast maltase, oligo-1,6-glucosidase, and dextranase; four in maltotetraohydrolase, neopullulanase, *Bacillus stearothermophilus* pullulanase, and isoamylase; five in cyclodextrin glucanotransferases, branching enzymes, and maltogenic α-amylase; and six or more domains in α-amylase-pullulanase, *Klebsiella aerogenes* pullulanase, maltopentaose, producing α-amylase, and *Bacillus polymyxa* β-amylase-α-amylase. At least 10 different types of domains have been suggested (Jespersen *et al.*, 1991), but a functional role has been assigned only for the three different kinds of catalytic domains (Matsuura *et al.*, 1984; Buisson *et al.*, 1987; Boel *et al.*, 1990; Kubota *et al.*, 1990; Mikami *et al.*, 1990; Klein & Schulz, 1991; Aleshin *et al.*, 1992; Kadziola *et al.*, 1992) and for the so-called starch-binding domain, described first as the C-terminal part of glucoamylase from *Aspergillus niger* (Svensson *et al.*, 1982, 1983) and later recognized as being evolutionary related to domains in amylolytic enzymes of diverse specificity (Svensson *et al.*, 1989): *Streptomyces* α-amylases; a thermostable β-amylase; maltogenic α-amylase; maltotetraohydrolases; *Aspergillus* glucoamylases; and cyclodextrin glucanotransferases. The ability of this domain to adsorb onto starch has been used in genetically produced chimeric enzymes aiming at facilitated downstream processing (Chen *et al.*, 1991). Detailed studies on the isolated domain, prepared from *Aspergillus niger* glucoamylase by limited proteolysis catalysed by subtilisin (Belshaw & Williamson, 1990), have shown that it contains an extended binding site with specificity for cyclic and linear maltooligodextrins (Belshaw & Williamson, 1991). While the affinity of this domain was especially good for the different cyclodextrins, the potent glucoamylase inhibitor acarbose was less firmly bound at the C-terminal domain, in contrast to the ligand specificity seen for the active site on the catalytic domain (Svensson & Sierks, 1992). With respect to the other domains found in amylolytic enzymes, they are as yet of unknown function except for a small one (so-called B-domain) grafted onto the catalytic domain as a long polypeptide loop of the α/β-barrel framework. Only in the case of loops at the C-terminus of the third β-strand in α-amylases has structural evidence been

obtained for participation in the binding of substrate and structural Ca^{2+} (Matsuura *et al.*, 1984; Buisson *et al.*, 1987; Boel *et al.*, 1990). The sequence alignment of α/β-barrel domains from related enzymes guided by the prediction of secondary structural elements furthermore proposed that certain enzymes hydrolysing α-1,6-linkages in contrast have a relatively small loop following the third β-strand, while an additional long loop was predicted to extend from a different β-strand (Jespersen *et al.*, 1991, 1993). One may speculate, therefore, that accommodation in the active site of a branched substrate in analogy to the modelled complex of Taka-amylase A and maltoheptaose would be facilitated by a relatively small loop at the position of the so-called B-domain, while further stabilization of complexes between the enzymes in question and branched substrates could be provided by loops elsewhere at the barrel rim (Jespersen *et al.*, 1991, 1993). The tentative outline of the domain level organization in addition suggested a correlation between the action in endo-fashion on α-1,6-glucosidic bonds and the occurrence of structural domains N-terminal to the catalytic domain (Jespersen *et al.*, 1991). The sequential order of domains along the polypeptide chain may vary as has been seen, for example, in the case of the starch granule binding domain that succeeds the catalytic domain in the *Aspergillus* glucoamylases and precedes the catalytic domain in the *Rhizopus* glucoamylases (Svensson *et al.*, 1989). A similar variation in domain sequential order has been found also in different cellulolytic enzymes (Knowles *et al.*, 1987). It may thus be a structural feature characteristic of enzymes that attack insoluble polysaccharide substrates. Since the isolated domains have retained the ability to adsorb onto such substrates (Belshaw & Williamson, 1990; Reinikainen *et al.*, 1992) they may primarily secure a firm attachment of the enzyme. At the same time a long, flexible linker region between domains provides possibility for spatial adjustment of the relative position of the extended active site cleft to the part of the polysaccharide chain susceptible to hydrolysis. Despite this functionally important multidomain architecture, specific sites for adsorbtion onto granular starch are not necessarily restricted to separate structural domains, as has been demonstrated for example for the α-amylase from barley (Gibson & Svensson, 1987; Søgaard & Svensson, unpublished).

In barley AMY2 and porcine pancreatic α-amylase it was attempted to separate the structural domains by limited proteolysis (Desseaux *et al.*, 1991). The α-amylases are generally quite resistent to proteolysis, but specific cleavage was achieved under rather harsh conditions. The C-terminal domain, however, was not split from the barrel, whereas cleavage of specific peptide bonds occurred in the two enzymes in the seventh and the eighth loop, respectively, extending from the C-terminus of the corresponding β-strands of the barrel. This indicates an intimate association between the different structural domains in α-amylases. The only indication of the C-terminal domain being critical for the enzymatic function came from random mutagenesis in the enzyme from *Bacillus stearothermophilus*, where loss of activity resulted from a number of mutations in the C-terminal domain (Holm *et al.*, 1990).

SUBSTRATE BINDING AND CATALYSIS

Typical protein-substrate interactions have been described first for a modelled complex of the Taka-amylase A and a seven glucosyl residues long segment of amylose modelled in the active site on the basis of the difference Fourier map made for the crystals soaked with maltotriose (Matsuura *et al.*, 1984). For porcine pancreatic α-amylase a different Fourier map has been made for a complex with a maltotriose analogue bound in the catalytic site and the protein groups interacting with the substrate analogue have been listed (Buisson *et al.*, 1987). Preliminary information on a few substrate binding residues has been reported for β-amylase (Mikami *et al.*, 1990; Uozumi *et al.*, 1991) and several side chains in cyclodextrin glucanotransferase from *Bacillus stearothermophilus* are described to be engaged in ligand binding (Kubota *et al.*, 1990). Also a number of residues from the C-terminal domain of that enzyme that is homologous to the starch granule binding domain in *Aspergillus* glucoamylase have been found to participate in ligand binding. As is the case for all other enzymes having an α/β-amylase fold (Farber & Petsko, 1990), the presumed substrate binding and catalytic residues in α-amylases and cyclodextrin glucanotransferases are located at the C-terminal ends of barrel β-strands and in the loops extending from these strands connecting to succeeding α-helices. Amylases and other enzymes that interact with polymeric carbohydrate substrates possess extended binding sites that have the capacity to accommodate up to about 10 glycosyl residues. In the case of Taka-amylase almost all the loops at the C-terminal ends of the β-strands of the α/β-barrel are implicated in binding of the amylose chain extending over seven binding subsites (Matsuura *et al.*, 1984). Kinetic analysis allows the mapping of the binding affinities for glycosyl residues associated at each individual subsite of the substrate binding area (Robyt & French, 1963; Hiromi *et al.*, 1983). In pancreatic α-amylase subsite mapping thus revealed five binding subsites (Seigner *et al.*, 1985). In the barley α-amylases, however, a longer substrate binding cleft has been indicated (MacGregor & MacGregor, 1985). Recently more detailed analysis of the barley α-amylase isozyme forms, AMY1 and AMY2-1 and AMY2-2, two members of the AMY2 family, indicated 10 subsites, four towards the reducing and six towards the non-reducing end from the bond to

be cleaved. The extreme subsite towards the non-reducing end of the substrate chain had a very high affinity (Ajandouz *et al.*, 1992). The 80% sequence identity between these isozymes may allow modelling of the AMY1 isozyme based on the recently determined three-dimensional structure of AMY2 (Kadziola *et al.*, 1992) and a comparison between these two structural models may allow correlation of the differences in affinity at specific subsites with structural differences. This may open up possibilities of engineering the action pattern of amylases and thus changing the product compositions (see Svensson & Søgaard, 1993, for a recent review). Because of results from the structure determination and modelling of different α-amylase substrate complexes, we are at the beginning of understanding how local structural features influence the affinity for individual glycosyl residues and hence control the productive binding mode of the substrate. Typically aromatic groups are involved in protein-carbohydrate interactions and may secure a relatively strong interaction (Quiocho, 1986, 1989). This kind of interaction has thus been recognized in complexes of amylolytic enzymes such as Taka-amylase A (Matsuura *et al.*, 1984), porcine pancreatic α-amylase (Buisson *et al.*, 1987), *Aspergillus* glucoamylase (Clarke & Svensson, 1984; Sierks *et al.*, 1989), and cyclodextrin glucanotransferase (Kubota *et al.*, 1990). A property, particular probably to all cereal α-amylases, is a second binding site located outside the active site region. This site has affinity for granular starch and in barley α-amylase cycloheptaamylose competes with the binding onto the granules (Weselake & Hill, 1983). By differential labelling using cycloheptaamylose to protect reactive tryptophanyl residues against modification with Koshland's reagent we thus identified Trp 276 and Trp 277 in barley AMY2 to be involved in starch granule binding, while protection with an inhibitor, aplanin (DP 12), that includes the acarbose pseudotetrasaccharide core, prevented inactivation and chemical modification of Trp 206 in the active site (Gibson & Svensson, 1987).

The catalytic mechanisms of glycosylases is a key discussion issue. There is thus still debate on which are the important events during catalysis in hen egg white lysozyme, one of the best studied glycosylases (Strynadka & James, 1991; Lumb *et al.*, 1992). One may distinguish in the first place between retaining and inverting glycosylases, i.e. whether they release their product with either the same or the opposite anomeric configuration at the newly formed reducing end, as found in the cleaved substrate bond. Well-known examples of inverting enzymes among the amylolytic enzymes are glucoamylase and β-amylase, but most of the starch degrading enzymes hydrolyse glucosidic bonds with retention of the anomeric configuration. In that case two possible mechanisms are discussed, the S_N1 and the S_N2 mechanism. The former involves an electrostatically stabilized oxocarbonium ion intermediate, while the latter is characterized by formation of a covalent glucosyl-enzyme intermediate involving an enzyme nucleophile, either an Asp or a Glu residue. The first event in all three mechanisms is the protonation of the glycosidic oxygen of the bond to be cleaved by the catalytic general acid, found to be either an Asp, a Glue or a Tyr residue. A review describes the mechanism of different glycosylases in great detail (Sinnott, 1990). The utilization of mutational analysis to investigate the function of glycosylases (Svensson & Søgaard, 1993) and the prospects for protein engineering of amylases (Svensson & Søgaard, 1991) and other carbohydrate metabolizing enzymes (Teeri, 1991) have been addressed in different short reviews. With regard to the α-amylases and the related enzymes it is generally believed that they work by an S_N2 mechanism (Tao *et al.*, 1989; Sinnott, 1990; Svensson & Søgaard, 1993). It was hoped that site-specific mutagenesis could be a useful tool for insight to be gained into the specific functional roles of the three carboxylic acid and two histidine residues that invariably occur at the central part of the active site cleft. In the following a summary will be given of our own work with establishing a system for production of recombinant barley α-amylase AMY1 and AMY2, and the properties of the obtained mutants.

BARLEY AMY1 AND AMY2

Inside the barley grain the mature endosperm tissue contains large and small starch granules and storage proteins which are mobilized during the germination. This utilization is dependent on hydrolases that are either *de novo* synthesized in the aleurone cell layer as regulated by plant hormones produced during the germination or present already in the mature seed. Two gene families encoding different forms of α-amylase isozymes respond to the hormone gibberellic acid (Jones & Jacobsen, 1991). The two isozyme families of the barley α-amylase AMY1 and AMY2 have, despite the high sequence identity of 80%, several characteristic and distinctly different properties with respect to both enzymic action, stability, and sensitivity to thiol reagents and the endogenous proteinaceous inhibitor barley α-amylase/subtilisin inhibitor (BASI). Other enzymes such as limit dextrinase (Lee & Pyler, 1982) and different α-glucosidases (Stark & Yin, 1987) are produced as well. *In vivo*, all of these enzymes are believed to act in concert with the β-amylase in the endosperm. Extensive synergy in the initial attack on starch granules has been described in in-vitro model experiments (Maeda & Nikuni, 1978). The actual liberation of the form of β-amylase that dominates in malt (Lundgard & Svensson, 1987) is likely to be due to a limited proteolysis releasing it from a short C-terminal

region which may be linked to other proteins through a disulphide bridge. In brewing, the kilned malt ready for mashing already has extensively modified starch granules. Channels filled with amylolytic enzymes criss-cross the large granules, while a good part of the small granules has completely disappeared. In the mashing step, agitation and heating promote the enzymic destruction of the granule structure to soluble oligodextrins. Theoretically they may be completely broken down to glucose by the available enzymes, but normally the main products are fermentable malto-oligodextrins, accompanied by small amounts of higher (usually branched) oligosaccharides and some resistent starchy material. Ultimately, protein engineering of the endogenous amylolytic enzymes and genetic control of their availability may provide a way to tailor the dextrin profile according to specific requirements for production of different types of beer.

Even though the strategy for production of mutants of barley α-amylases AMY1 and AMY2 (Fig. 1) seems straightforward, it still is a long procedure. First the plant cDNAs (Rogers & Milliman, 1983; Rogers, 1985) encoding a member of each of the AMY1 and AMY2 isozyme families were going to be expressed in a system that secured correct processing to mature forms and produced sufficient amounts for characterization of mutant enzymes. We chose yeast as a host, since active recombinant wheat (Rothstein *et al.*, 1987) and mouse salivary (Thomsen, 1983) α-amylases had been produced previously in this organism. The vector pMA91 containing the phosphoglycerate kinase promoter and terminator gave the best result in expression of AMY1 and AMY2 (Søgaard & Svensson, 1990). Both of the recombinant isozymes were secreted to the medium, as mediated by their endogenous signal peptides, greatly facilitating the further purification in a simple affinity chromatography step. Unfortunately, only one of the isozymes, AMY1, was produced in a reasonable amount, that is 1–2 mg/litre. The deletion of a poly dG/dC region from the 5′-non-coding region of the AMY1 cDNA was necessary for obtaining the high yields. This is probably due to the fact that polyG RNA forms strong secondary structures impeding the work of the ribosome. AMY2 at the best was obtained at about 0·05 mg/litre (Søgaard & Svensson, 1990). Various attempts to arrive at a higher rate of heterologous protein synthesis by similar changes in the cDNA encoding AMY2 have failed. Among these attempts were also the complete replacement of the non-coding regions of the cDNA encoding AMY2 with the suitable non-coding regions of the cDNA encoding AMY1. However, because of recurring low amounts of recombinant AMY2 we decided to perform the site-directed mutagenesis of residues located to the active site and the starch granule binding site, respectively, in AMY1, in spite of the fact that the crystals suitable for X-ray crystallography were obtained with AMY2 (Svensson *et al.*, 1987) and that AMY2 is the dominant family in malt. As a last resort to improve the level of expression *in vivo*, recombination in yeast of the two cDNAs was pursued in collaboration with Jean-Claude Chaix and his group. The current results achieved with that strategy are reported by Juge *et al.* (1992). We hope to get reasonable amounts of recombinant AMY hybrids that comprise the major part of AMY2 and in this way be able to assign various isozyme specific properties to specific parts of the AMY2 molecule by biochemical studies of the recombinant AMY1/AMY2 hybids.

1. Construction of cDNA expression plasmid
2. Yeast transformation
3. Yeast fermentation (1, 10, 100 litre)
4. Supernatant work-up:
   (a) AMS ppte
   (b) cycloheptaamylose-Sepharose
   (c) Fractogel CM 650M
   (d) DTT treatment

Fig. 1. Strategy for preparation of AMY1 mutants.

The AMY1 and AMY2 produced in the heterologous expression system were readily isolated by affinity chromatography on cycloheptaamylose-Sepharose. Characterization of the enzymic and chemical properties indicated a slight decrease in activity for AMY1 and that the molecular weight and the N-terminal sequence appeared identical to the enzymes purified from malt (Søgaard & Svensson, 1990). As a routine the purity criteria for AMY1 and AMY2 from malt included determination of the isoelectric points (Svensson *et al.*, 1985), the simplest way of distinguishing the members of the two isozyme families. Surprisingly the recombinant AMY1 gave rise to four major bands with distinctly different isoelectric points (Fig. 2), while recombinant AMY2 migrated as a single dominant band and a faint band of slightly higher pI (Søgaard & Svensson, 1990). Moreover, the corresponding zymograms developed by iodine staining after incubation in a starch solution gave a very clear qualitative picture, that two of the four AMY1 forms had low specific activity compared to malt AMY1. In essence only one of the four AMY1 bands showed both pI and activity similar to malt AMY1. In order to use the AMY1 expression system also for AMY1 mutants it is important to understand the mechanisms leading to the four different forms and next to be able to make a single recombinant AMY1 form. Previous observations in barley aleurone cell cultures revealed a precursor-product relationship for AMY1 forms, where precursors of higher pI were residing mainly intracellularly and processed forms were found in the medium (Jacobsen *et al.*, 1988). At that time the change in isoelectric point was examined for sulphation, phosphorylation, glycosylation and acetylation, but none of these post-

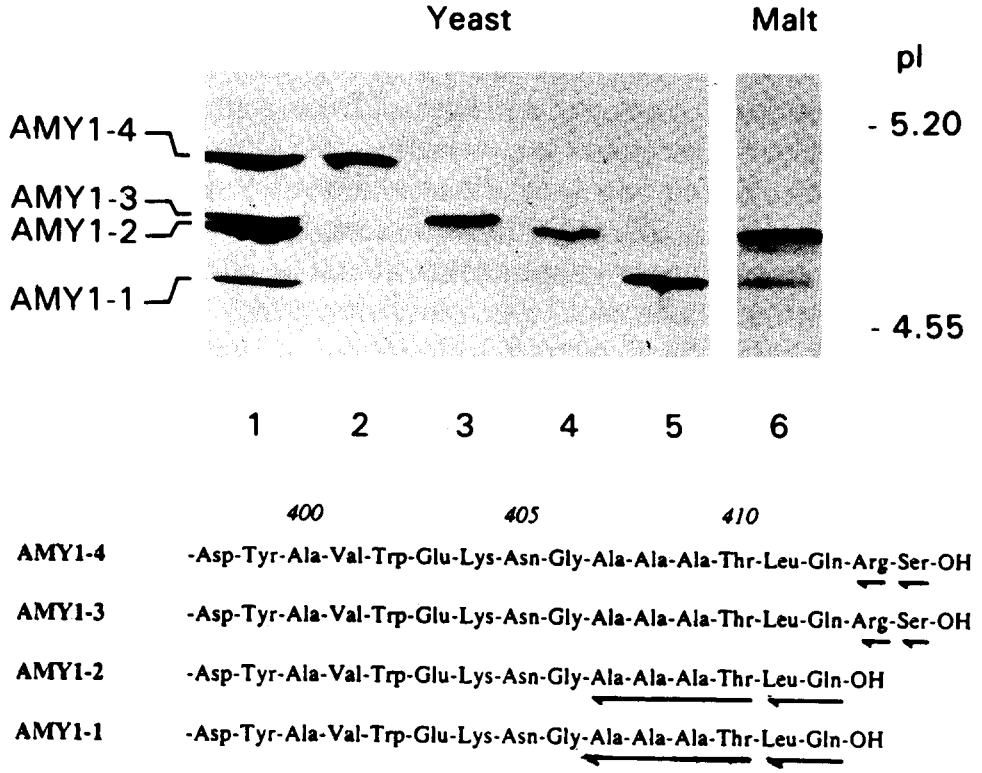

Fig. 2. Isoelectric points and *C*-terminal sequences of recombinant forms of barley low pI α-amylase AMY1-1 (lane 5), AMY1-2 (lane 4), AMY1-3 (lane 3), and AMY1-4 (lane 2). Recombinant AMY1 prior to separation (lane 1) and AMY1 prepared from malt (lane 6) are included for comparison. (Modified after Søgaard *et al.*, 1991.)

translational reactions could be demonstrated to take place. Alternatively, processing of the polypeptide chain could be due to proteolysis or deamidation. The latter type of modification is extremely difficult to identify when only a small amount of protein is available, and since the processing of the signal peptide was correct as demonstrated by N-terminal sequencing, it was tested whether carboxypeptidase treatment might change the AMY1 forms. The pI values were certainly affected, our four forms being reduced to AMY1-2 and AMY1-1, the two forms with the lowest pI values (Søgaard *et al.*, 1991). Both yeast carboxypeptidase Y and malt carboxypeptidase II efficiently catalysed the reaction. For chemical analysis of the structural changes, a 100 litre fermentation of recombinant AMY1 was made. The pure forms were separated by ion exchange chromatography, following affinity chromatography. C-terminal sequence analysis indicated that the dipeptide Arg-Ser had been removed from AMY1-4 and AMY1-3 with generation of AMY1-2 and AMY1-1, respectively (Fig. 2). *In vitro*, isolated AMY1-4 and AMY1-3 could be processed into AMY1-2 and AMY1-1, respectively, by carboxypeptidase treatment. Thorough examination of their amino acid compositions gave the clue to the reason for the low activity of AMY1-3 and AMY1-1, which were found to have one Cys modified by glutathione γ-Glu-Cys-Gly. Thus somehow misfolding and improper pairing of the SH groups seemed to be the cause for the low activity. This was confirmed by treatment with dithiothreitol prior to isoelectric focusing, which changed the AMY1-3 and AMY1-1 forms into AMY1-4 and AMY1-2, respectively, due to the loss of the negatively charged glutathione. Also direct detection of glutathione on the amino acid analyser after reduction was in accordance with these results. Malt AMY1 is believed to contain one disulphide bridge and two free thiol groups (Søgaard *et al.*, 1992); AMY2 contains only three SH groups (Gibson & Svensson, 1986). Titration of the free SH with DTNB, however, indicated that the recombinant AMY1-2 and AMY1-4 have four SH groups. Subsequent molecular weight analysis by electrospray mass spectrometry of the four forms showed mass differences matching exactly those represented by the loss of Arg-Ser and the addition of glutathione, thus confirming these post-translational modifications. C-terminal sequence analysis of AMY1 purified from malt indicated a further processing leading to loss of the seven C-terminal residues probably catalysed by several malt carboxypeptidases of different specificity (Søgaard *et al.*, 1991). In an attempt to obtain a single recombinant form of AMY1, Cys 95 present in AMY1, but not in AMY2 (Rogers & Milliman, 1983; Rogers, 1985), was replaced by Ala and the C-terminal dipeptide Arg-Ser was removed by genetic truncation. When this mutated cDNA was expressed in the yeast system only one

product appeared. Using the truncated wt gene led to AMY1-2 and AMY1-1 and treatment with dithiothreitol resulted in just AMY1-2, which had essentially the same enzymic properties as the malt AMY1. C-terminal processing in yeast is due to Kex1 protease, a Golgi-localized serine carboxypeptidase, since expression in a yeast strain deficient in this protease led to production of only AMY1-4 and AMY1-3 (Søgaard *et al.*, 1991).

The question arose whether the AMY1 precursor forms in aleurone cells were degraded by malt carboxypeptidase(s) in a physiological process to the dominant AMY1 gene products. To address that question the AMY1 patterns in lysates of aleurone cell protoplasts (cv. Himalaya) and in the culture media were monitored by the isoelectric focusing-zymogram technique (Søgaard *et al.*, 1991). In the cell lysates precursors were clearly present for what seemed to be, dependent on the harvest, two or three pairs of AMY1 forms. In the media essentially only the processed forms were observed. Addition of inhibitors of serine carboxypeptidase, however, suppressed the conversion of the putative precursor forms, lending support to the hypothesis that *in vivo* C-terminal processing also occurs as catalysed by endogenous carboxypeptidase. Evidence for C-terminal processing was also obtained by treating AMY1 purified from protoplast lysates with carboxypeptidases, thereby chasing precursors into products. The functional significance of this conversion remains to be understood. Recently attempts to identify the subcellular site of the processing led to the conclusion that the conversion takes place all the way from the endoplasmic reticulum through the Golgi apparatus and after secretion (Sticher & Jones, 1992).

AMY1 MUTATION

In order to investigate the specific functional roles of conserved carboxylic acid and histidine residues at or near the site of catalysis in α-amylases and related amylolytic enzymes (MacGregor, 1988), the following residues in AMY1 were subjected to site-directed mutagenesis: His 93, Asp 180, Glue 205, His 290 and Asp 291. The acids were replaced by the corresponding amides and the histidines by asparagine (Fig. 3). In addition, Trp 278 and Trp 279 previously identified to interact with cycloheptaamylose at the surface site (Gibson & Svensson, 1987) and assumed to be involved in the binding onto starch granules (Weselake & Hill, 1983), were replaced in a double and two single mutations by alanine residues.

The fermentation yields greatly varied dependent on the mutant. Thus essentially wild type level was obtained for Asp291 → Asn, while 10% of that level was obtained for Asp180 → Asn and Glu205 → Gln, and only 1% was obtained for His93 → Asn, His290 → Asn, and the Trp279 → Ala. It was necessary therefore to

ACTIVITY ON PHADEBAS BLUE STARCH	
His93-Asn	5%
Asp180-Asn	0%
Glu205-Gln	0%
His290-Asn	10%
Asp291-Asn	0%
Trp279-Ala	22%

Fig. 3. Effect of mutation of active site and surface site residues in barley low pl α-amylase.

produce the latter mutant in a 100 litre fermentation. The Trp278 and the double mutants were not produced. All mutants were secreted to the culture medium and could be purified by affinity chromatography. The activity was tested using *p*-nitro-phenylmaltoheptaoside, amylose (DP 17), and Phadebas blue starch as substrates. Substitution of either of the three carboxylic acid groups resulted in total loss of hydrolytic activity (Fig. 3). Attempts to further characterize these mutants included evaluation of the affinity for acarbose-Sepharose. The mutants clearly bound less tightly to the column as compared to the wild type enzyme. Difference spectroscopy for acarbose binding to both wild type and these AMY1 mutants on the other hand gave a K_d value in the range of 1 mM. This is approximately 100-fold higher than the K_i determined for the wild type AMY1, indicating two binding sites for acarbose in AMY1, the second site probably being the known surface binding site with affinity for cycloheptaamylose. The replacement of either of the two well-conserved histidyl residues gave mutants with 5% and 10% of the wild type activity on Phadebas blue starch (Fig. 3). The effect of the specificity constant (k_{cat}/K_M), however, was much more pronounced on small than on large substrates, suggesting that these histidyl residues contribute to productive binding (Søgaard & Svensson, unpublished). In the modelled complex of maltoheptaose and Taka-amylase A (Matsuura *et al.*, 1984), both of the corresponding residues His 122 and His 296 interact with the glucosyl residue in the glycon part of the substrate adjacent to the bond to be cleaved. The present results indicate that the histidines are important for transition state stabilization and not in the proton-transfer by the general acid catalyst to the glucosidic oxygen of the bond to cleaved. Further support for this conclusion was provided by the fact that the affinity for acarbose, assumed to be a transition state analogue, had decreased in the mutants by about the same factor as found for the decrease in the value for the specificity constant k_{cat}/K_m. The pK_2 was determined from the pH rate profile for both amylose and *p*-nitrophenylmaltoheptaoside to be about 6·5 which is very similar to the value determine for wild type AMY1. Essentially the

same conclusion was arrived at by mutational analysis of the functional role of some of the five invariant active site residues in other α-amylases and related enzymes (Holm *et al.*, 1990; Vihinen *et al.*, 1990, 1991; Nakamura *et al.*, 1992; Kuriki *et al.*, 1991; Takase *et al.*, 1992; Nagashima *et al.*, 1992).

Trp279 → Ala, the mutant obtained at the chemically identified surface site, had 22% of wild type activity towards Phadebas blue starch and similarly reduced activities towards amylose and *p*-nitro-phenylmaltoheptaoside. There is thus no discrimination on the effect of the active site between small and large soluble and insoluble substrates, respectively. This suggests that cooperation between the two binding areas on the enzyme surface is not important. The present mutant, however, has 10-fold lower binding affinity for barley starch granules (K_d of approximately 2 mg/ml) than wild type AMY1 (K_d approximately 0.2 mg/ml) (Søgaard & Svensson, unpublished). The K_d for cycloheptaamylose, reported to compete with barley starch granule binding, was also increased by the mutation. Clearly, although the mutant retains some affinity at the binding site, the strength has been considerably reduced compared to the wild type AMY1. The tryptophanyl side chain is therefore an important, but not an essential structural part of the binding site.

ROLE OF Ca^{2+} AND BASI FOR AMY ACTIVITY

AMY2 is much more sensitive to Ca^{2+} than is AMY1 (Bertoft *et al.*, 1984). The crystal structure of AMY2 has been found to contain three Ca^{2+} atoms (Kadziola *et al.*, 1992). We therefore reinvestigated the influence of the Ca^{2+} on the activity of two isolated forms of AMY2, AMY2-1 and AMY2-2, and AMY1, respectively. Characteristically the hydrolytic activity of the AMY2 forms on blue starch was raised about 2–4-fold, with the $CaCl_2$ concentration increasing from 1 to 15 mM. The AMY1 activity in this range remained essentially constant at the level similar to the activity of AMY2 at about 0·5 mM $CaCl_2$. The activity of all isoforms dropped dramatically from 15 to 40 mM $CaCl_2$. Similar inactivation has been explained in the case of α-amylase from *Aspergillus niger* to be due to Ca^{2+} interacting with carboxylic acids at the catalytic site (Boel *et al.*, 1990).

Another property that distinguishes the AMY1 and AMY2 isozyme families is the sensitivity to the endogenous proteinaceous α-amylase/subtilisin inhibitor (BASI) which is present in the mature barley seed (Mundy *et al.*, 1983; Weselake *et al.*, 1983). In a screening for functional residues required for the inhibitory activity of BASI, the effect on activity of chemical modification with group specific reagents was examined. Modification of eight arginines out of twelve present in BASI (Svendsen *et al.*, 1986) led to complete inactivation (Abe *et al.*, 1993*b*). Only partial inactivation resulted from specific treatment of carboxylic acids, tyrosines, and histidines, while primary amino groups, methionines and tryptophans appeared not to be involved in the inhibitory activity. In an approach to identify the functionally important groups a differential labelling experiment was performed. AMY2 was utilized for protection of BASI during modification of exposed arginyl residues with phenylglyoxal. Following dissociation of the modified AMY2–BASI complex and isolation of the active BASI, which had four modified arginines, remodification of the BASI derivative by phenylglyoxal led to loss of inhibitory activity concomitant with the modification of four additional arginyl residues (Abe *et al.*, 1993*b*). The identification in the sequence of the arginines protected by AMY2 is ongoing. Since the three-dimensional structure has been reported of the inhibitor from wheat having 94% sequence identity to BASI (Zemke *et al.*, 1991) candidates for essential residues have been pointed out. These are currently subjected to site-directed mutagenesis performed in the cDNA encoding BASI, for which a yeast expression system has recently been established in our laboratory (Abe *et al.*, 1993*a*). The yield of correctly processed recombinant BASI was reasonable when using the yeast invertase signal peptide for secretion.

SUMMARY

We have described here recent observations and findings from our laboratory involving four topics all related to the degradation of starch in cereals. Firstly, it was outlined how the enzymes involved in starch degradation with different specificities in fact belong to a few structural families described as the α- and the β-amylase families, the glucoamylase family and perhaps a fourth family including certain α-glucosidases. Secondly, we have briefly indicated important functional and structural features of α-amylases in general, and thirdly we have described active site and starch granule binding site mutants of the barley malt α-amylase AMY1, including the establishment of a heterologous expression system. Finally we have touched upon work embarking on the study of protein–protein interactions in the barley AMY2–BASI complex.

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