Chapter 11

Chromatography as the Major Tool in the Identification and the Structure-Function Relationship Study of Amylolytic Enzymes from Saccharomycopsis Fibuligera R64

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1. Introduction

Chromatography, at both preparative and analytical levels, has been a key experimental technique in the study of proteins, primarily liquid chromatography in the separation, purification, and analysis. Recent developments in the study of proteins lean towards simplification and miniaturization, thus chromatography becomes less involved and explored. For example, development of protein tags and their associated affinity matrices enables purification of a protein in one step. This chapter describes the identification and structure-function relation study of amylolytic enzymes from *Saccharomycopsis fibuligera* R64, where liquid chromatography was used as the major tool, not only in the purification but also biochemical analysis of the enzymes.

2. *S. fibuligera* R64 secretes amylolytic enzyme

S. fibuligera is a food-borne yeast that is widely used in the production of rice- or cassava-based fermented food, i.e. *Tape* in Indonesia and other Southeast Asia countries (1). The yeast, in combination with *Saccharomyces cerevisiae* or *Zymomonas mobilis*, has been employed in the production of ethanol using cassava starch as the starting material (2), where the starch is firstly degraded into simple sugars prior to (bio-) ethanol. Bioethanol has been promoted as a renewable energy replacing fossil fuels and at the moment is already used as an additive. As the demand for renewable energy grows, *S. fibuligera* emerges as an attractive workhorse for the bio-ethanol production.

Degradation of starch into sugars is performed by amylolytic enzymes, such as α-amylase, glucoamylase, β-amylase, isoamylase, pullulanase, exo(1-4)-α-D-glucanase, α-D-glycosidase, and cyclomaltodextrin-D-glucotransferase (Fig. 1) (3). *S. fibuligera* secretes amylolytic enzymes, almost exclusively α-amylase and glucoamylase. α-Amylase acts as an endo-enzyme, cleaving 1,4-α-glycosidic bond at random positions to result in liquefaction of starch. Glucoamylase, on the other hands, is an exo-enzyme that cleaves 1,4-α-glycosidic bond only at the non-reducing end to result in saccharification (4). Thereby upon the combined action of α-amylase and glucoamylase, starch is degraded into maltose, maltotriose, or dextrin, and subsequently hydrolyzed to glucose.

The use of amylolytic enzymes for the ethanol production in the course of renewable energy requires an ability to act on raw starch, allowing the use of biomass as the starting material. The ability of *S. fibuligera* α- and glucoamylase to degrade raw starch has been reported (4, 5). Interestingly, only 10% of amylolytic enzyme-secreting organisms are capable of producing raw starch degrading α-amylase (6, 7). Since starch degradation begins with α-amylase action to produce simpler sugars, raw-starch acting α-amylase is highly desired. This situation strengthens the position of *S. fibuligera* for bioethanol production, being a raw starch degrading enzyme producer.

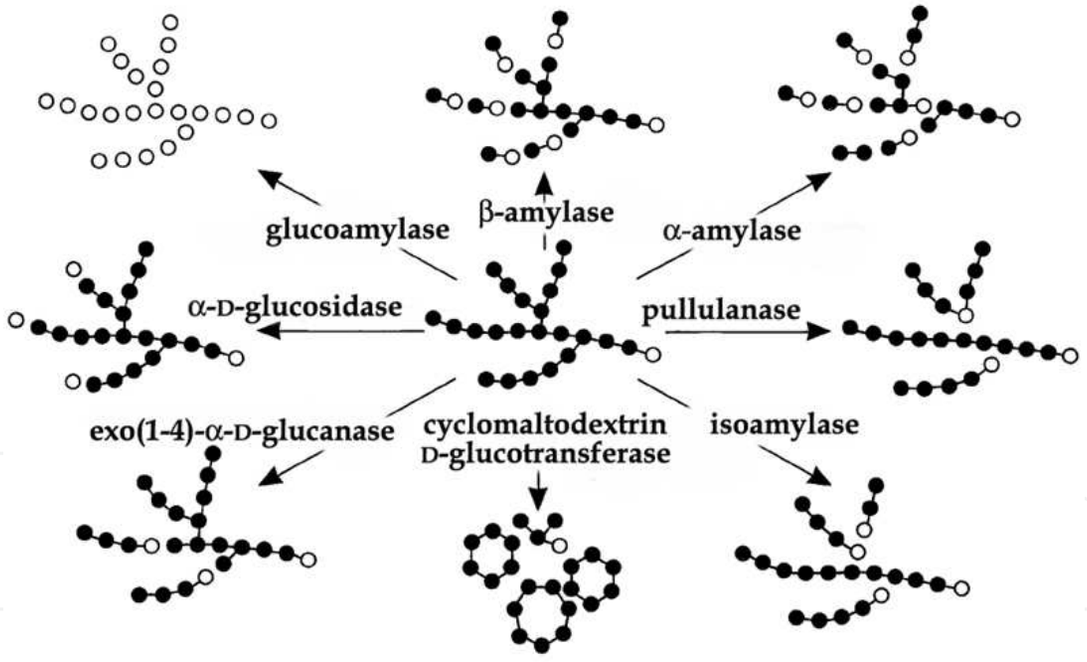

Figure 1. Starch degradation by amylolytic enzymes (3). The open and black coloured circles represent the reducing and non-reducing sugars, respectively. Note that the cleavage occurs at the reducing sugar.

α-Amylase is commonly used in food, beverage, paper industries (8), in textile industry and as additive in detergents (9, 10), for renewable energy (11, 12) and medical purpose (13). Glucoamylase has been the most important enzyme in food industry, mainly in the production of sugar or ethanol (14). Glucoamylase is normally employed in combination with amylolytic enzymes that are able to act on more complex polysaccharides, such as α-

amylase and pullulanase (14). Recently, amylolytic enzymes are employed in the production of lactic acid and ethanol by-product by lactic acid bacteria (LAB) (15), demonstrating that the application of amylolytic enzymes continues to expand.

Interestingly, numbers and characteristics of amylolytic enzymes secreted by *S. fibuligera* vary from one strain to another. For example, strain IFO 0111 secretes only glucoamylase whilst strain KZ secretes α-glucosidase along with α-amylase and glucoamylase (4). Further, glucoamylases from strain IFO 0111 and HUT 7212 demonstrate raw starch degradation whereas that from the strain KZ does not. Glucoamylase from strain KZ, however, demonstrates better thermal stability (4). Fascinating to find these variations in their properties despite of their near identical acid sequences (16). Strain KJ-1, an *S. fibuligera* strain from Indonesia, is also reported to secrete only glucoamylase and its partial amino acid sequence (residues 28-47) is identical to that of glucoamylase from strain KZ (1). This observation indicates differences in expression and secretion of amylolytic enzymes between *S. fibuligera* strains. Nevertheless, the practicality of *S. fibuligera* in producing amylolytic enzymes applauds the proposal to sequence its whole genomic DNA (2).

Of 136 isolates screened from various places in Indonesia, *S. fibuligera* strain R64 was selected for demonstrating the highest amylolytic activities (α-amylase and glucoamylase). Strain R64 is able to degrade raw starch and its amylolytic enzymes demonstrate potent thermal stability. This finding makes the amylolytic enzymes from strain R64 attractive.

Extra-cellular amylolytic enzyme production by strain R64 is relatively simple, using a medium that contains of 1% sago starch and 1% yeast extract. The enzyme was harvested after 4-5 days of cultivation in a one-litre bioreactor, with constant aeration rate 1 vvm, volumetric oxygen transfer coefficient (k_{La}) 1.53 per hour, agitation speed 100 rpm, 30^{o}C, and pH 7.0. Under this condition, enzyme activity observed on starch degradation was 1320 U/ml (Table 1). The production was also easily reproduced at a laboratory scale by means of Erlenmeyer flask (5). Thus, strain R64 offers a simple but convenient production scheme.

3. Isolation of the amylolytic enzyme complex

The amylolytic enzyme complex from strain R64, consisting of α-amylase (AMY) and glucoamylase (GLL1), is secreted into the production medium. Thus, the enzyme complex was harvested by simply cold-centrifugation (~4^{o}C) at 6000 *g* for 30 minutes, to remove the yeast cells. The enzyme complex in the supernatant, which was designated as the crude extract, was subjected to a diafiltration system (Millipore Minitan II, Tangential Flow Filtration system, Merck Pte Ltd, Singapore) over a 10-kDa cut off membrane disc-plate, at a flow rate of 10-20 ml per minute, at room temperature. The enzyme complex was recovered in the retentate. Diafiltration step tremendously reduced the size of the sample, which is advantageous because the subsequent step to capture the amylolytic enzyme complex in the crude extract was precipitation with ammonium sulfate by 0-100%, on ice (~4^{o}C). These sequential procedures demonstrate a straightforward way to isolate extra-cellular proteins that was accomplished within one-day operation, which is important for protein works due to, for example, possible degradation by proteases.

4. Purification of AMY and GLL1 in chromatography columns

Since the final step in the isolation procedure in the pilot experiment was precipitation with high concentration of ammonium sulfate, subsequent purification on a size-exclusion chromatography (SEC) column appears to be the most appropriate approach because it also functions as a desalting procedure. SEC column requires minimum size of sample upon application (recommended less than 3-5% of the column volume), which can easily be overcome by dissolving the protein precipitate from ammonium sulfate precipitation in a small volume. Another option is purification using an anion or cation exchanger chromatography (abbreviated as AEX and CEX, respectively) columns. However, this either type of column requires the removal of salts prior to sample application. This requirement can be countered by either diluting the sample until the conductivity of the sample solution is low or by dialysis against a buffer that contains low salt concentration.

The ammonium sulfate precipitate containing AMY and GLL1 was immediately dissolved in a small portion (3-5 ml) of 25 mM Tris-HCl buffer, pH 8.0. Unfortunately, AMY and GLL1 were not separated in an SEC column as suggested from the enzyme activity assays. In a sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS PAGE) analysis, AMY and GLL1 was not separated and appeared as one protein band with a molecular mass of 55±2 kDa. Further, purification in a DEAE-cellulose AEX column has also failed to separate the two enzymes. This finding indicated a similarity in the distribution of charge on the surface of AMY and GLL1. In addition, there was a recommendation to avoid the use of carbohydrate- based column material for the purification of proteins with an affinity towards carbohydrate, because of the possible interaction between the protein and the supporting column matrix (17). Thus, both SEC and AEX columns were obliterated from the separation of AMY from GLL1.

As purification strategies based on either protein size or charge failed to separate AMY from GLL1, exploiting differences of their protein surface hydrophobicity profiles emerged as an alternative. This strategy was tested on a hydrophobic interaction chromatography (HIC) column, where the purification proteins is based on the hydrophobic character of the surface of the proteins (18). In an HIC column, proteins in solution are conditioned with high salt concentration, which enforces interaction between proteins and the hydrophobic resin. Separation of AMY from GLL1 in an HIC column is lucrative because it can be carried out immediately after precipitation with high concentration of ammonium sulfate. Thus, the use of the HIC column is complementary to the developed isolation procedure.

4.1. Separation of AMY and GLL1 in an HIC column

The ammonium sulfate precipitated protein was dissolved in 25 mM Tris-HCl buffer, pH 8.0, containing 25% ammonium sulfate (w/v). This protein solution was applied to a butyl-Toyopearl 650M HIC column (Tosoh Bioscience Corp., Tokyo, Japan), which had previously been equilibrated with the same buffer. The column was then eluted with 25 mM Tris-HCl buffer, pH 8.0, containing a decreasing concentration of ammonium sulfate (25-0% of 5%

decrement, w/v). The separation profile of AMY and GLL1 in the HIC column is presented in Fig. 2. AMY was eluted at the ammonium sulfate concentration of 15% (w/v) whilst GLL1 was at 5% (w/v). This result demonstrated that AMY and GLL1 were successfully separated based on their hydrophobicity. Moreover, the elution profile suggests that the surface character of GLL1 is more hydrophobic than AMY.

Purification of proteins in HIC column is influenced by the pH of the solution where hydrophobic interaction is stronger at lower pH (19). This means, less hydrophobic proteins are bound stronger onto the HIC matrix at lower pH value. This phenomenon may explain different separation profile of AMY and GLL1 observed upon purification in an HIC column using phosphate-citrate buffer system at pH 5.8 (5), where α-amylase activity was detected in the GLL1 fraction. Although this hypothesis is yet to be proven, the separation at neutral to basic pH (above 7.0) is recommended for optimum operation.

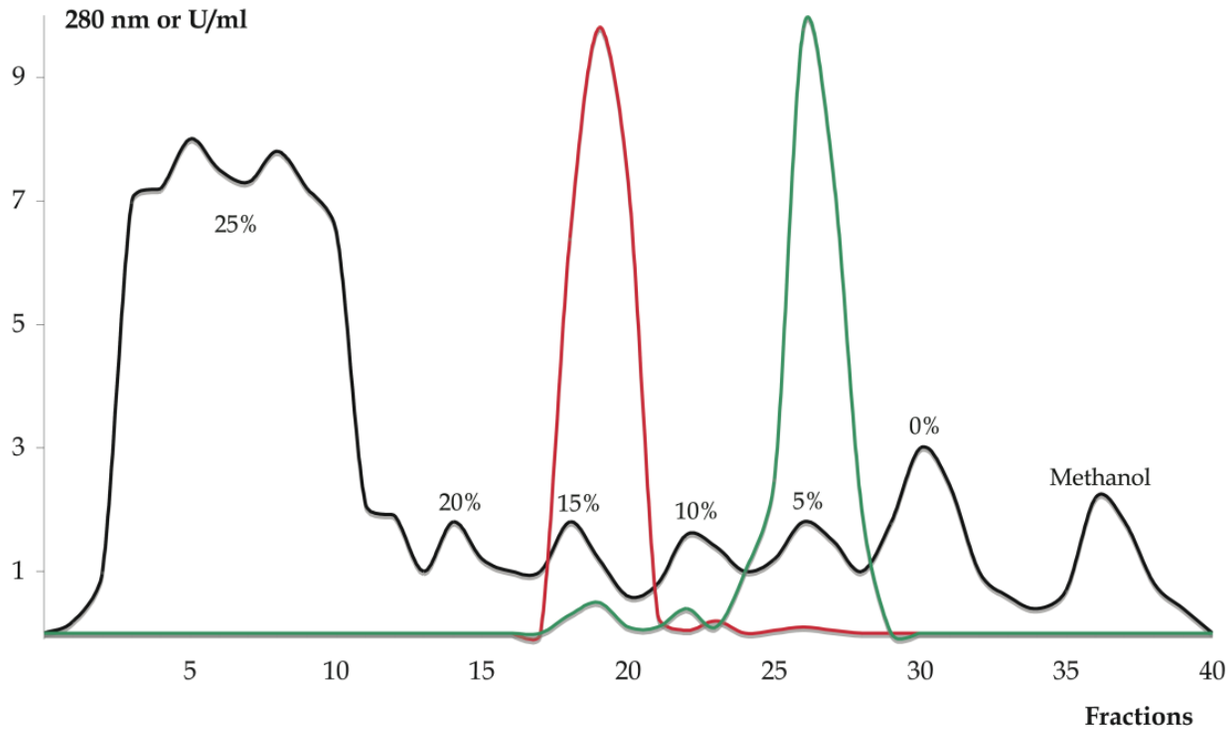

Figure 2. Separation profile of AMY and GLL1 in the butyl-Toyopearl 650M HIC column. The black, red, and green lines represent the protein absorbance at λ 280 nm, α-amylase activity (X 500 U/ml), and glucoamylase activity (X 10 U/ml), respectively. α-Amylase activity was based on starch degradation (20) whilst glucoamylase was on reducing sugar (21).

The use of HIC column to separate *S. fibuligera* α-amylase from glucoamylase was reported previously with an amylolytic enzyme preparation from strain KZ (22). That attempt was performed using a Spheron 300 LC HIC column, following successful separation of hog's pancreas α-amylase from its amylolytic enzyme complex (23). However, unlike butyl-Toyopearl column where proteins are captured by the butyl ligand, hydrophobic interactions in a Spheron column occur between the proteins and the hydrophobic backbone of the matrix, similar to separation of proteins in a reversed-phase high performance liquid

chromatography (RP-HPLC) column. In the Spheron 300 LC column, α-amylase (strain KZ) was eluted at an ammonium sulfate concentration of 10% whilst glucoamylase was at 5%. The separation profile, however, is strikingly similar to that of AMY and GLL1 with butyl-Toyopearl column. However, separation of AMY from GLL1 in the butyl-Toyopearl column has better resolution, suggesting variations in the surface properties of *S. fibuligera* α-amylase and glucoamylase from different strains, or the butyl-Toyopearl resin serves for better separation because there is no interaction between the proteins and the supporting matrix. Nevertheless, these results emphasize the power of HIC to separate α-amylase from glucoamylase.

4.2. Subsequent purification of AMY or GLL1 in chromatography columns

Intended for their characterization, AMY and GLL1 were independently collected and subjected to subsequent purification with DEAE-Toyopearl 650M AEX column. An SDS PAGE analysis showed that these purification steps resulted in pure AMY (5) and GLL1 (16). The final purification scheme of AMY is summarized in Table 1. Further, whilst the presence of two and three types of α-amylase and glucoamylase, respectively, were reported upon purification of these amylolytic enzymes from strain KZ by a Mono-Q anion exchanger column (22), AMY and GLL1 appeared to be the only amylolytic enzyme species from strain R64. Nevertheless, additional analysis was performed to confirm that the isolation of purified AMY and GLL1 was unanimous.

	Volume (ml)	Total activity (Units)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Crude	2000	2640000	16500	160	100
25% AS	2200	2244000	13046	172	85
Butyl Toyopearl	148	1082400	753	1438	41
95% AS	35	765600	213	3595	29
DEAE-Toyopearl	60	580800	100	5808	22

Table 1. The final purification scheme of AMY. AS stands for ammonium sulfate.

4.3. Identification of AMY and GLL1 using RP-HPLC for protein

Nowadays, identification of proteins usually employs techniques with sophisticated and delicate instrumentation e.g. peptide-mass finger print mass spectrometry (PMF-MS) (24). Unfortunately, this technique is rather pricey and furthermore, requires convenient access to the protein database and to the amino acid sequence of the protein. Particularly in the lack of the latter, which is a very common situation in the early stage of protein works, the separation of AMY from GLL1 was confirmed by a more simple and robust technique, exploiting the use of an RP-HPLC system (25). The analysis using RP-HPLC is based on the fragmentation profile of a protein after proteolytic digestion. Proteolytic digestion of AMY

was expected to result in fragmentation profile that differs to that of GLL1, as reflected from their chromatographic profiles.

The analysis of AMY and GLL1 by RP-HPLC was carried out essentially following Soedjanaatmadja and co-workers on the identification of pseudo-hevein from the latex of rubber tree (26). Firstly, purified AMY was incubated for four hours at 37°C with chymotrypsin (EC. 3.4.21.1), at an AMY to chymotrypsin mass ratio of 100:1, in 200 mM ammonium bicarbonate buffer, pH 8.0. The reaction was stopped by an addition of 10 mM hydrochloric acid, to lower the pH of the solution. Preparation of GLL1 sample was done in the same way, including the substrate to chymotrypsin mass ratio.

The reaction mixture was immediately applied to an RP-HPLC nucleosil 10 C18 column (30 x 0.45 cm) and the separation was performed for 60 minutes, at a flow rate of 1 ml/min, using 0-70% acetonitrile gradient in 0.1% trifluoroacetic acid (TFA). Elution of fragments was monitored at λ 214 nm, which is specific for detection of peptide bonds. The fragments are eluted according to their hydrophobicity, where more hydrophobic fragments are retained longer in the RP-HPLC column.

Chymotrypsin cleavage takes place on peptide bonds at the C-terminal part of preferably tyrosine, phenylalanine, tryptophan, and leucine residues, and with (much) less extent of methionine, valine, isoleucine, histidine, glycine and alanine residues (27). Due to this broad specificity, the cleavage may result in many fragments varying in size and hydrophobicity, as observed in the chromatographic profiles of proteolytically digested AMY and GLL1 (Fig. 3).

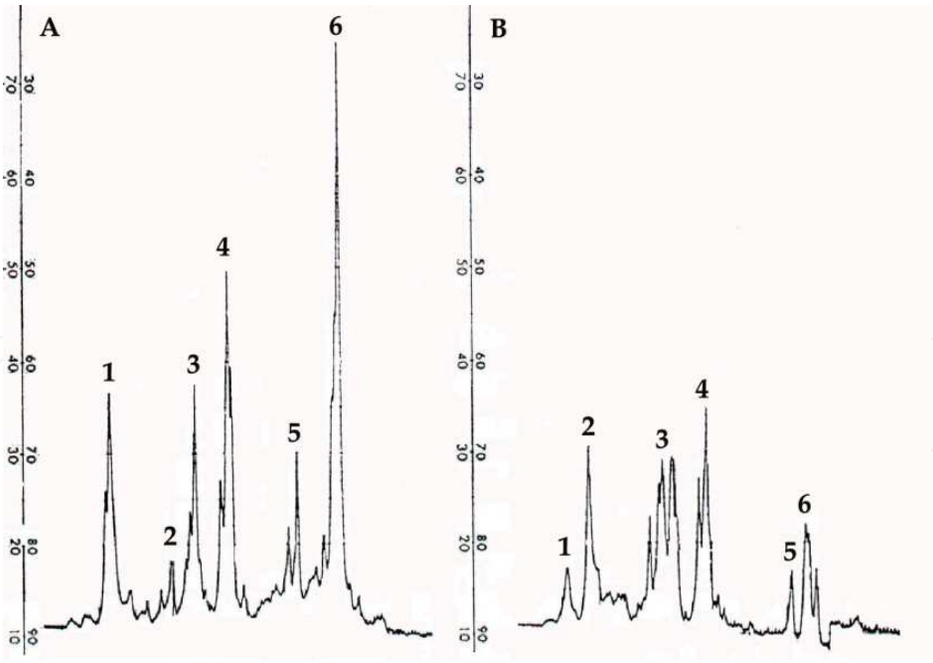

Figure 3. Chromatographic profile of fragments from proteolytic digestion AMY (A) or GLL1 (B) as monitored at λ 214 nm.

The fragmentation profile of AMY was significantly different from that of GLL1. Six sets of resolved peaks were recovered in both cases (Fig. 3) but their retention time, intensities, and

peak distribution were different. Intensity of the peaks suggested much less materials were recovered from GLL1 digested samples than from AMY. This situation is likely contributed from highly hydrophobic or negative charged fragments that were not eluted from the RP-HPLC column due to their poor solubility in the solvent used (28). The amino acid sequences of AMY and GLL1 (see 3.5) showed that the charged amino acid distribution in the amino acid sequence of GLL1 (100 charged amino acid residues out of 494, ~20.2%) is higher than that of AMY (86 out of 468, ~18.4%), thus AMY contains more hydrophobic and non-charged amino acids. Unfortunately, correlation between the result from RP-HPLC and hydrophobic amino acid distribution cannot firmly be established because the nature of chymotrypsin digestion was unclear. The results may indeed indicate that hydrophobic amino acids in GLL1 are likely more clustered than in AMY, resulting in highly hydrophobic peptide fragments in GLL1. This hypothesis may be related to the structure of GLL1 (Fig. 8), which consists of one globular molecule that opposes two separable domains of AMY. However, possibility for the existence of highly negative charged peptide fragments from GLL1 can also not be excluded. Nevertheless, of the eluted fragments (Fig. 3), majority of AMY fragments are localized at peaks 5 and 6 whilst GLL1 are at peaks 3 and 4, suggesting different fragmentation had occurred. Thus, the RP-HPLC profiles of AMY and GLL1 indicated successful separation of the two enzymes.

4.4. Analysis of starch hydrolysis products to discriminate AMY and GLL1

Glucoamylase hydrolyzes starch at the non-reducing end of amylose or amylopectin to result in glucose therefore the enzyme activity assay is normally based on the release of reducing sugar (21, 29). Unfortunately, α-amylase random digestion of starch may also result in reducing sugar i.e. maltose (30) therefore detection of glucoamylase activity in an α-amylase preparation can be anticipated. Glucoamylase can also, at lesser extent, act on 1,6-α-glycosidic bond of amylopectin (31), although its efficiency diverse greatly depending on the source organisms. Hydrolysis of the 1,6-α- bond may result in a less integrated starch molecule, hampering the formation of iodine-starch complex, which is the basis of standard α-amylase activity assay (20). Therefore, cross-detection of the two enzyme activity assays is inevitable. This phenomenon was notorious upon separation of AMY from GLL1 at pH 5.8 (5), where α-amylase activity was detected in GLL1 fractions.

One approach to discriminate α-amylase and glucoamylase is *via* the evaluation of their hydrolysis product. α-Amylase action results in various kind of oligomeric sugars (32) whilst glucoamylase hydrolysis product is glucose (monomeric). Based on this principle, successful separation of GLL1 from AMY was assessed through their hydrolysis products, as analyzed with Thin Layer Chromatography (TLC) (16), using purified GLL1 that was obtained from the purification procedure carried out at pH 5.8.

Purified GLL1 was incubated with soluble starch substrate at 37°C and samples were taken after 5, 10, 15 and 45 minutes of incubation. Each sample was then applied onto a silica gel 60 TLC plate (20 cm x 20 cm, Merck, Darmstadt, Germany) using capillary glass tube. The plate was then placed in a TLC separation chamber that had been equilibrated with the

mobile phase, which was the mixture of butanol : ethanol : water (at a ratio of 5:5:3, v/v/v). The plate was retrieved from the TLC separation chamber after the mobile phase migration reached three quarters of the length of the plate and then immediately short-submersed (few seconds) in a mixture of water : methanol : sulphuric acid (at a ratio of 45:45:10, v/v/v). The plate was then heated at 120°C for 15 minutes on a hot plate for visualization of the sugars.

The TLC profile (Fig. 4) shows that the product of starch hydrolysis by GLL1 was solely monosaccharide i.e. glucose (G1). The intensity of the G1 spot was increasing from 5 to 45 minutes of incubation, indicating that glucose was produced over time. The spots at the sample application points were from the un-hydrolyzed soluble starch substrate. Apparently, most of the starch molecules were hydrolyzed within 15-45 minutes of incubation. Most importantly, no higher oligomeric sugar forms were detected, even during the first 5-10 minutes of incubation, suggesting the absence of α-amylase. This result provided the undisputed proof that GLL1 had successfully been separated from AMY despite of α-amylase activity was detected in the GLL1 fraction. Thus, the detected α-amylase activity was not originated from AMY.

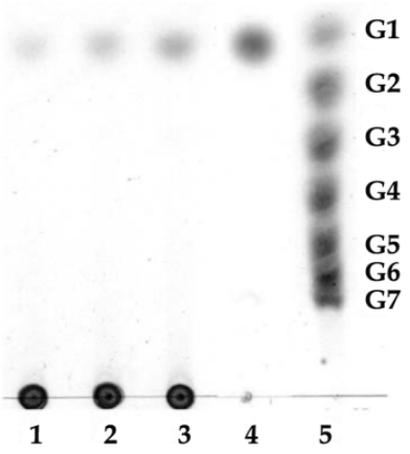

Figure 4. Analysis of starch hydrolysis products of GLL1 on a TLC plate (16). Lane 1-4 is hydrolysis products after 5, 10, 15, and 45 minutes, respectively. Lane 5 is the mono- (G1), di-(G2), and oligo-saccharide (G3-G7) markers.

4.5. Properties of AMY and GLL1

Since the purity of AMY and GLL1 was firmly established, each enzyme could now be characterized. AMY was found active at a pH range of 5.0-7.5 and a temperature range of 30-60°C, with an optimum at 5.5 and 50°C, respectively (5). GLL1 was also found active a pH range of 4.6-6.8 and a temperature range of 30-65°C, with an optimum at 5.6-6.4 and 50°C, respectively (16). These results suggest that the two enzymes are active at similar conditions. Moreover, their characters are similar to most other *S. fibuligera* amylases.

Recently, the amino acid sequences of AMY (GenBank accession code HQ172905) and GLL1 (HQ415729) were successfully elucidated as derived from their chromosomal DNA (16, 33).

The amino acid sequence of AMY is similar to that of published earlier by Itoh and coworkers (34), having mutations at six amino acid residues (Asp>Asn153, Ile>Val159, Ser>Asn190, Ser>Xaa216, Asp>Asn239, and Ser>Thr295). The six deviating residues in AMY comprise an additional predicted glycosylation site (Asn153), which according to a structural modelling, is highly plausible because it resides in a long surface loop (33).

The amino acid sequence of GLL1 is similar to glucoamylases from strain HUT 7212 (GLU) and KZ (GLA) (16). GLU and GLA share high sequence identity (4), with only seven amino acid residues different. However, these seven residues are responsible for differences in their characteristics, where GLA exhibits potential thermal stability and GLU has higher affinity towards substrate. The amino acid sequence of GLL1 also differs to both GLU and GLA at precisely those particular seven residues, which four are being identical to GLU and three to GLA. Interestingly, these mutations result in GLL1 to adopt the potential thermal stability and higher affinity towards substrate. Thus, GLL1 behaves as a hybrid of GLU and GLA.

The calculated theoretical pI values (35) of AMY and GLL1 (based on the amino acid sequences of their mature protein sequences: AMY sequence starts at residue Glu27 of the full-length protein containing signal and pre-peptides as encoded by *AMY* gene whilst GLL1 sequence starts at residue Ala1 as reported in the data base) were 4.4 and 4.3, respectively. The isoelectric point (pI) value of AMY was confirmed experimentally, being 4.6±0.2. This finding supports the early observation that AEX or CEX columns are unable to separate the two enzymes. Similarly, the calculated theoretical molecular masses of AMY and GLL1 were also similar, being 51.7 kDa and 54.6 kDa, respectively. Both values were confirmed experimentally, being 54±2 kDa for AMY and 56±2 kDa for GLL1. Additional ±2 kDa of AMY and GLL1 masses is likely contributed from glycosylation. This result also confirms the inability of SEC column to separate them.

The activity of AMY is diminished in the presence of ethylene diamine tetra acetate (EDTA), a chelating agent. Inactivation of α-amylase by chelating agent is well known, as the enzyme requires calcium ion for its activity and integrity (36). This inactivation by chelating agent was not observed with GLL1. However, the activities of both AMY and GLL1 were increased in the presence of calcium or magnesium ions (data not shown). Further, AMY demonstrated lower activity in phosphate-citrate buffer. This phenomenon may arise from the citrate, which is reported to interact with calcium ion (37). The activity of AMY decreased concomitantly with the increase of the citrate buffer concentration but was fully recovered upon back-titration with calcium chloride (data not shown). Based on this observation, citrate buffers (1, 5) should only be used with considerable reservation and the use of tris buffer is recommended. This recommendation is in line with the necessity to perform purification at basic pH for HIC. In addition, this finding may also explain higher glucoamylase activity detected in AMY fractions when the purification was carried out using phosphate citrate buffer, pH 5.8 (5). Since the buffer does not influence the glucoamylase activity as it does to AMY, the disparity between the two enzyme activities was much less pronounced in comparison to the one presented in Fig. 2.

The hydrophobicity profile of AMY and GLL1 was analyzed based on the amino acid sequence of the mature enzymes (38), using an online analysis service at ExPASy (http://web.expasy.org/protscale/) (35). Another handful online service is also available at http://www.vivo.colostate.edu/molkit/hydropathy/index.html. The window size values for the frame normalization were scanned from 3 to 21 and compared to determine the significance of the regions that represent the hydrophobic character. The profile at window frame 9 (recommended for hydrophilic protein) and 19 (for hydrophobic protein) are presented in Fig. 5. The prediction points were linearly weighted with 100% relativity at the window edges. The score for hydrophobicity is ranged from -4.5 (hydrophilic) to 4.5 (hydrophobic), where the curve above the midpoint (zero) is interpreted as regions with hydrophobic character. As shown in Fig. 5, regions with hydrophobic character in AMY are at the residues 50-75, 210-230, 310-330, 375-410, and 425-440, whilst for GLL1 are at 225-230, 375-380, 425-445, and 475-480. This *ab initio* results suggest that AMY has more fragments with hydrophobic character, which may also be due to the presence of two separable domains in AMY that each has its own hydrophobic core. However, this result may not be corresponded with RP-HPLC profile as the fragmentation of AMY and GLL1 upon digestion with chymotrypsin occurs randomly, where more hydrophobic fragments could be produced from GLL1.

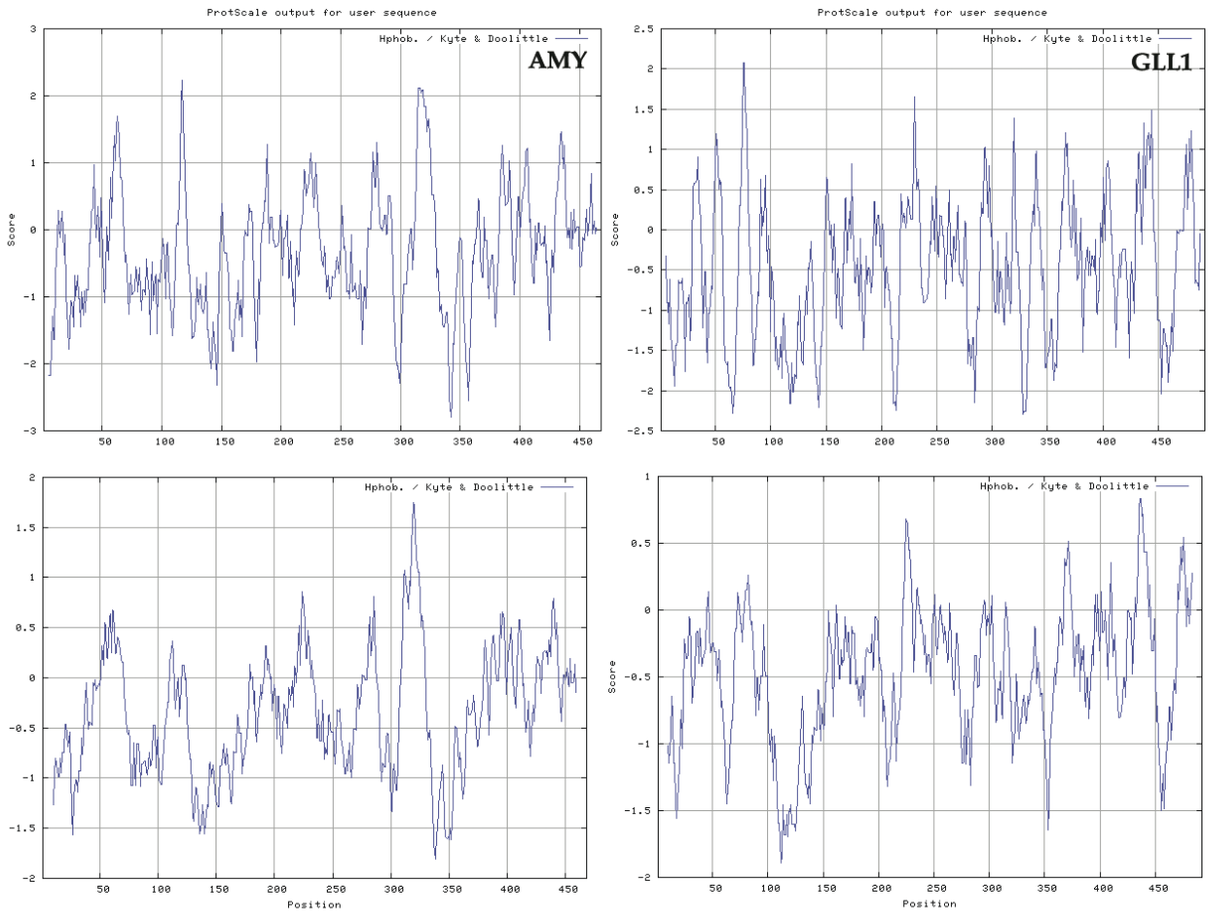

Figure 5. Hydrophobicity profile of AMY and GLL1 as calculated according to their amino acid sequences (38). The upper graphs are produced with window frame of 9 whilst the lower was with that of 19.

Another computational study was performed using the on-line hydrophobic cluster analysis (HCA) program (39). This approach has previously been done to compare the hydrophobic clusters in α-amylases (40). The sequence of AMY and GLL1 were submitted to the drawcha server (http://bioserv.impmc.jussieu.fr/hca-form.html) and the resulted profiles were analyzed manually following Gaboriaud *et al.* (41). The profiles were manually mismatched with the structures of AMY (*in silico*, generated from its homolog, see section 5) and GLU, correspondingly. AMY was predicted to have 34 hydrophobic clusters whilst GLL1 was 17. However, only 3 hydrophobic clusters of AMY are exposed on the protein surface as oppose to 7 of GLL1. The analysis suggests the presence of more hydrophobic patches on the surface of GLL1 than on that of AMY. Thus the result corresponded with the experimental finding with HIC column.

5. AMY recombinant behaviour on purification in chromatography columns

The biochemical characteristics of AMY and GLL1 are to be improved to meet specific conditions for application, such as resistance to high temperature, chemical inactivation, and proteases (42). This can be achieved by engineering at both gene and protein levels, which require convenient access to the genetic information and protein structure. Although the genes encoding for AMY or GLL1 are successfully elucidated, commencing an educated and directed genetic engineering entails the structure of the enzymes. The amino acid sequence of GLL1 is nearly identical to that of GLU (43), which its structure has been reported (in complex with amylases inhibitor acarbose, PDB accession code 2F6D). Therefore, structural study of GLL1 was performed employing the structure of GLU. The structure of *S. fibuligera* α-amylase is, on the other hands, not available. The structural study of fungal α-amylase has been employing the structure of the enzyme from *Aspergillus niger* (PDB code 7TAA) (44), which shares only 35% homology to AMY (5). Although the use of that structure is amenable, it might not be able to describe the details of characteristics of AMY. Therefore, elucidating the structure of AMY became a priority. Leading to this aim, heterelogous expression of AMY in *Escherichia coli* was attempted.

Overexpression of soluble and functional AMY in *E. coli*, with a His_6-tag at its N-terminus for easy purification on a Nickel-agarose affinity column, was unsuccessful. This situation was not improved after change of the vectors that harbour the AMY gene and of *E. coli* strains, and manipulation of overproduction conditions. No amylolytic activity or protein band at the expected molecular mass upon an SDS PAGE analysis was detected in the cell lysate. Overexpression of proteins from higher organisms in bacterial system often results in the formation of inclusion bodies because of the lack of post-translational machinery (45). As α-amylase is a glycoprotein (46), which requires post-translational modification for its expression, this lack of that machinery was an obvious suspect. Actually, was AMY overexpressed successfully in *E. coli* as soluble protein, attempt leading to structure determination would be easier because post-translational modifications are reported to hamper protein crystallization (47), the initial step for structure determination by means of

X-ray crystallography. Nevertheless, based on the result of overexpression, the attempt for heterelogous expression of AMY in *E. coli* was withdrawn for the use of expression system from higher organism i.e. yeast *S. cerevisiae*.

S. cerevisiae has been a popular choice for heterelogous expression of proteins (48) and tested to express α-amylase and glucoamylase from *S. fibuligera* strain HUT 7212 (49, 50). *S. cerevisiae* expression system for expression of *S. fibuligera* α-amylase and glucoamylase has also been improved, i.e. the enzymes expression was drastically increased under the control of the *S. cerevisiae* constitutive phosphoglycerate kinase (PGK) promoter (51). Motivated from that success, AMY was overexpressed in *S. cerevisiae* INVSc1, using yEP-secretex vector and galactose as the inducer. However, although AMY recombinant demonstrated similar activity to AMY, its molecular mass is substantially higher, being 67±2 kDa. Unfortunately, no such information was provided from the expression of *S. fibuligera* α-amylase from strains HUT 7212 (50) and Eksteen (51). Since no polypeptide chain or protein tag was added, different glycosylation profile was likely the reason for the increase in the molecular mass of the recombinant protein. The finding of a plausible additional glycosylation site in the amino acid sequence of AMY applauds this proposal.

5.1. AMY recombinant behavior upon purification in AEX columns

Likewise AMY, AMY recombinant was secreted into overproduction medium therefore it was harvested by cold centrifugation at 6000 *g* for 30 minutes to remove the yeast cells. The enzyme was captured from the medium by fractionation with ammonium sulfate at a saturation degree of 0-100%. The ammonium sulfate protein precipitate was dissolved in 50 mM Tris-HCl buffer, pH 8.0 (~5 ml) and then dialyzed overnight against one litre of that respective buffer in the cold room (~4^{o}C).

The dialyzed AMY recombinant was then loaded onto a resource-Q AEX column (GE Healthcare Europe GmbH, Diegem, Belgium), which had been equilibrated with that respective buffer. Purification was performed in a cold cabinet (~7^{o}C) using a fast protein liquid chromatography (FPLC) Äkta system (GE Healthcare Europe GmbH, Diegem, Belgium) and monitored on-line with the Unicorn program. The enzyme was recovered from the column upon an elution with an increasing gradient of sodium chloride 0-1 M. As the control, purified AMY was also subjected to purification with the same column.

Purification of AMY in the resource-Q column showed that minor contaminants were still present in the purified sample (Fig. 6). The major contaminant has, however, higher absorbance at λ 260 nm, suggesting that it may not be protein. This contaminant appeared yellowish in colour, which may be originated from the overproduction medium component that was co-purified in HIC, AEX, and SEC columns. Further, the elution profile of AMY recombinant was very similar to that of AMY, except for an additional large protein peak upon sample application and washing step prior to the sodium chloride salt gradient. These additional peaks unmistakably are originated from other proteins and components of overproduction medium because the AMY recombinant sample applied was not purified prior to this column. AMY recombinant was not pre-purified with the HIC because no

glucoamylase was co-produced. Nevertheless, similarity of their elution profiles in the resource-Q AEX column suggests that AMY and AMY recombinant share similar surface charge distribution.

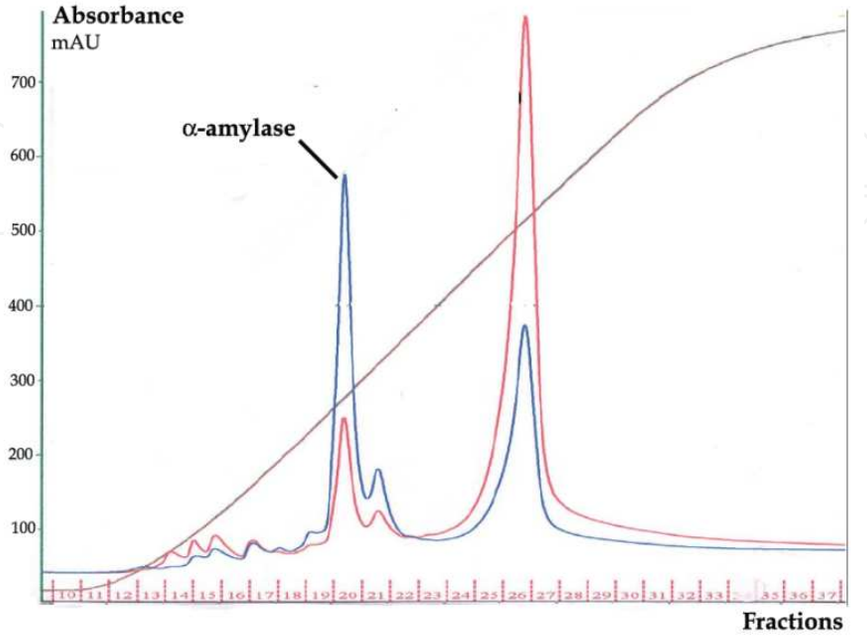

Figure 6. Elution profile of AMY on a resource-Q AEX column. The blue, red, and brown lines represent absorption at λ 280 nm (protein), at λ 260 nm, and the conductivity (mS/cm).

Further, AMY recombinant was subjected to purification in DEAE-52 cellulose AEX column (Whatman Nederland BV, s'Hertogenbosch, The Netherlands) following the purification of *S. fibuligera* α-amylase strain HUT7212 (46). Although DEAE cellulose have failed to separate AMY from GLL1 in the previous attempt, the binding of AMY to this AEX resin at purification conditions similar to that of Matsui's was observed. Thus, AMY recombinant should produce a comparable elution profile, as demonstrated previously during its purification on the resource-Q AEX column.

Supernatant from cold centrifugation was mixed with DEAE-52 cellulose AEX resin that had been equilibrated with 50 mM sodium acetate buffer, pH 5.2. After 1.5 hours of incubation at 4°C, the resin was allowed to settle and the unbound proteins were carefully decanted. The resin was washed three times with and then suspended in the respective buffer. The DEAE-52 cellulose AEX suspension was poured into an empty chromatographic column and eluted with the respective buffer. The proteins were recovered from the column by a sodium chloride salt gradient 0-1 M.

Surprisingly, AMY recombinant was not bound to DEAE-52 cellulose AEX resin, as judged from both SDS PAGE analysis and enzymatic assay. Reflecting back to purification on the resource-Q AEX column at pH 8.0, AMY and AMY recombinant were eluted at 30-35% of B solution (1 M sodium chloride, thus 300-350 mM), suggesting that the enzymes were not strongly bound to the AEX matrix. Thus, to observe no interaction between AMY

recombinant and AEX resin at pH 5.2 is logical, although the pH is still higher than the pI of the enzyme. However, AMY demonstrated binding to DEAE-52 cellulose matrix in that condition, suggesting the recombinant protein has different protein surface character.

5.2. AMY recombinant behavior upon purification in sugar affinity columns

Another approach to purify AMY recombinant is the use of α-, β-, or γ-cyclodextrin (CD) columns, which was reported previously for the purification of amylolytic enzymes (52). CD is cyclic polymer of D-glycopyranosyl that is linked by α-D-(1-4) glycosidic bond and has no non-reducing or reducing ends. The α-, β-, or γ- variant of cyclodextrin matrices differs only on the number of glucose residue that builds up the dextrin ring, being six, seven, and eight, respectively (53). The protein target is bound to the interior of the CD molecule *via* hydrophobic interactions, since this interior part is less hydrophilic than its surroundings. From this perspective, CD columns have similar function to HIC column. CD also interacts with the raw starch affinity but not active sites of α-amylases (53), unlike pullulanase or cyclodextrin glycosyltranferase. Interestingly, the binding to CD column also occurs to the raw starch degrading but not adsorbing α-amylases. These reports showed that the purification of amylolytic enzymes on a CD column is based on their affinity towards substrate like matrix material.

The use of CD affinity columns is lucrative because the purification can directly be carried out after the harvesting step. After cold centrifugation, the supernatant that contained AMY recombinant was directly loaded onto α-, β-, or γ- CD affinity columns, which had already been equilibrated with 10 mM sodium acetate buffer, pH 5.5. After an extensive elution with the same buffer to remove unbound proteins, the column was eluted with 1% (w/v) α-, β-, or γ- CD in 10 mM sodium acetate buffer, pH 5.5, respective to the type of the column. Samples taken during the elution were subjected to an analysis with SDS PAGE. As the control, this purification procedure was also applied to the purified AMY.

Purification of AMY on the CD columns showed an equally strong interaction with both α- and β-CD matrices variant but weakly to γ-CD, as judged from the amount of AMY eluted from the respective CD column (Fig. 7). The result is in agreement with the reported use of CD column to separate α- from β-amylase from the amylolytic complex in higher plants (52). Surprisingly, AMY recombinant was not bound onto any of the CD matrices. This result set further doubt on the surface character of AMY recombinant as being different to AMY.

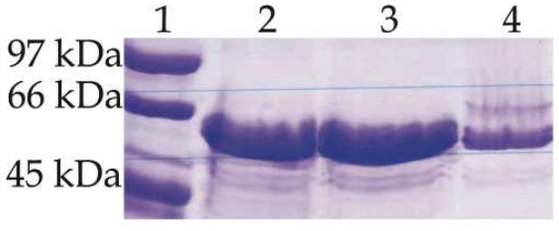

Figure 7. Elution of AMY from the α- (lane 2), β- (lane 3), and γ- (lane 4) CD columns. In lane 1 is the molecular mass marker.

Excessive amount of contaminants from the production medium was one of the suspects for this altered AMY recombinant behaviour upon purification with AEX DEAE-52 cellulose or CD columns, but the result from the latter challenged that possibility. Instead, different glycosylation pattern emerged as the prime suspect because glycosylation has indeed been reported as the main drawback for heterelogous expression of protein in *S. cerevisiae* (48). Hypermannosylation was detected upon the overexpression of *A. niger* glucoamylase in *S. cerevisiae* (54). Change of glycosylation profile may have negative impact as it can demolish enzyme activity or even change protein function (48). Furthermore, different glycosylation pattern may explain the observed higher molecular mass of AMY recombinant. Unfortunately, comparable information from similar works on the expression of *S. fibuligera* α-amylase from strains HUT 7212 (50) and Eksteen (51) was also not available.

Overexpression of amylolytic enzymes in *S. cerevisiae* is very attractive because it leads to direct conversion of starch to ethanol (11), for generation of renewable energy. Heterelogous expression of AMY in yeast *S. cerevisiae* has been successful to produce AMY recombinant, in term of enzyme activity. This AMY recombinant could be employed in further work leading to production of bio-ethanol. However, the purification profile of AMY recombinant differs substantially to AMY and the cause for this problem is yet to disclose. Therefore, the use of AMY is still preferred for the structure-function study.

6. Structure function study of AMY in the absence of the structure

The domain organization of AMY was studied by means of limited proteolysis by trypsin-TPCK, which has specific activity to cleave lysine and arginine residues. This procedure was employed to study the domain organization of bacterial cellulase (55). The fragments recovered from the limited proteolytic digestion of AMY were separated in a SEC column and subjected onto functional analysis (5).

Benefiting from the availability of amino acid sequences of AMY and GLL1, structural study *in silico* was performed (33). The structural model for AMY was prepared using the online model building program from the EMBL-EBI (56). Briefly, the amino acid sequence of AMY derived from its DNA (GenBank ID: HQ172905) was submitted to the online program SWISS-MODEL (57) using the structure of Taka-amylase from *A. niger* (PDB code 7TAA) as the template. The resulted model (7TAAmt) was evaluated using the program MolProbity (58), and manually assessed using the program *COOT* (59). The graphic representation was prepared with the program PyMOL (60), equipped with the programs PDB2PQR (61) and APBS (62) for the calculation of the surface charge distribution. As for GLL1, the structure of GLU was adopted because of near identical amino acid sequence.

The protein surface of AMY and GLL1 is mainly composed by negatively charged amino acid residues (Fig. 8). AMY does have more hydrophobic residues but they are concentrated at the interface between A/B to C domains. Discounting these domain interface hydrophobic residues in AMY, GLL1 has more hydrophobic patches on its surface. The large hydrophobic patch on the surface of AMY (Fig. 8, top right) is interrupted by positively

charged residues (lysine) and putative additional glycosylation site, which increases the overall hydrophilicity of that hydrophobic patch. The surface representation suggests that the surface profile of GLL1 is more hydrophobic, thus the *in silico* study supports results from the purification in HIC column. The overall negative charged residues on the protein surface might also explain the need for basic pH for the buffers used in the purification as well as the low pI of the two enzymes.

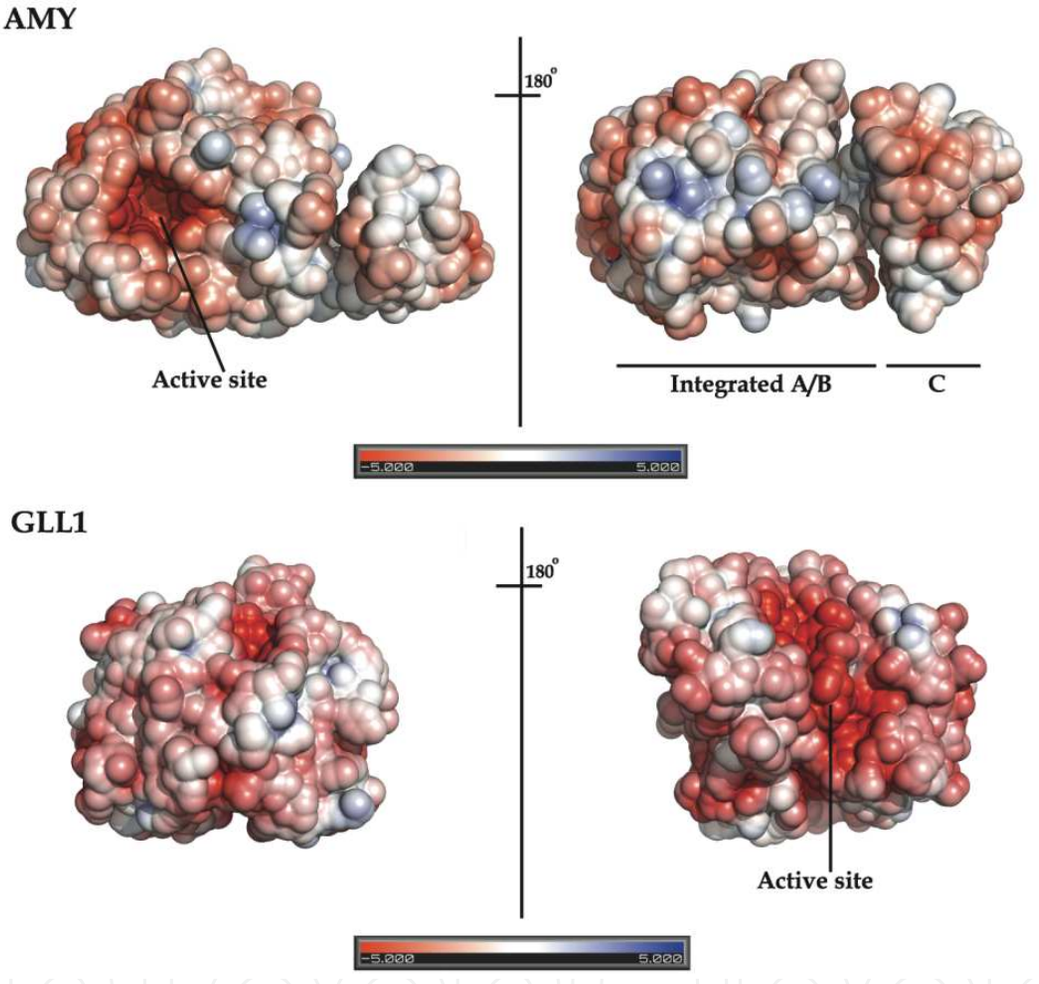

Figure 8. Surface charge distribution representation of AMY and GLL1. The red and blue colour represents negatively and positively charged amino acid residues, whilst the whitish are neutral of hydrophobic amino acids. The C domain of AMY is shifted away for clarity.

6.1. Proteolytic digestion of AMY

Purified AMY was incubated with trypsin treated with tosyl phenylalanyl chloromethyl ketone (TPCK) for 72 hours at 37°C at a substrate to protease mol ratio of 15:1. The reaction was carried out in 25 mM Tris-HCl buffer, pH 8.3 containing 20 mM calcium chloride. The reaction mixture was then transferred to -20°C for storage prior to further analysis in SDS PAGE analysis, or immediately applied to a sephadex G50 SEC column for the enzyme's domain separation (5).

Digestion of AMY by trypsin-TPCK resulted in two fragments with molecular masses of ~39 kDa (f39) and ~10 kDa (f10) (5), as judged from an SDS PAGE analysis. Based on the size of the fragments and proteolytic cleavage prediction according to its amino acid sequence, the f39 is designated as the N-terminal domain whilst f10 as the C-terminal. α-Amylases structure comprises of an $(\alpha/\beta)_8$-TIM barrel structural motif that is built up from the N-terminal part (domain A and B) and of the C-terminal part (domain C) (63). These two major domains are linked by a long surface loop. The integrated domain A/B is assigned as the catalytic domain whilst domain C is postulated as the starch-binding domain. As the two major domains of AMY were apparently separated upon proteolytic digestion, the functioning of f39 and f10 were evaluated.

6.2. Separation of f39 and f10 in an SEC column

The separation of f39 and f10 was performed in a Sephadex G50 SEC column (∅1.3 x 50 cm, bed volume ~48 ml) with gravity flow, in 20 mM phosphate citrate buffer, pH 5.8. Fractions of 5 ml were collected and the protein elution profile was measured by absorbance at λ 280 nm (UV-160, Shimadzu Corp., Tokyo, Japan). Only the collected protein absorption peak fractions were used for further analysis. As the control, purified AMY was also applied to the column and eluted with the same conditions for the proteolytically digested sample. The amount of AMY applied was also kept similar to that of used in the proteolytic reaction for fair comparison.

Two distinct protein peaks were recovered from the proteolytic digestion reaction mixture (Fig. 9) as oppose to one peak from the purified AMY. The use of SEC column at a 50 kDa cut off allowed a clear separation because AMY was eluted right at the end of the void volume retention whilst digested AMY was eluted after the void volume. Trypsin (~23 kDa) was not detected because its amount was very small (out numbered by f39 and f10, having an AMY to trypsin mass ratio of 34:1). Should trypsin be detected, it may contribute to a small increase of absorbance at fraction 7 of the digested AMY. Fractions 3 (of AMY), 4 and 8 (of f39 and f10, respectively) were selected for further analysis.

Only AMY and f39 demonstrated α-amylase activity, confirming the assignment of f39 function as the catalytic domain. However, the K_M value of f39 suggested lower affinity towards starch substrate. Interestingly, lower f39 K_M value was not followed by the decrease of the k_{cat} value of the reaction, suggesting that the catalytic efficiency was not disturbed (5). These observations further supported the assignment of f39 as the catalytic domain as well as suggested the function of f10 in substrate binding. Furthermore, the half-life time (IC50) value of f39 upon incubation at 50°C was also lower than that of AMY (5). This finding suggested that the absence of f10 also resulted in lower stability of f39. In conclusion, these findings served as an evidence for the proposed function of f10 to house the substrate recognition site (63) and to maintain thermostability (64) of α-amylase. This finding assigned AMY as a raw starch degrading but not-adsorbing enzyme. Ultimately, these results were confirmed by the independent group working on AMY homolog from *S. fibuligera* strain KZ (65).

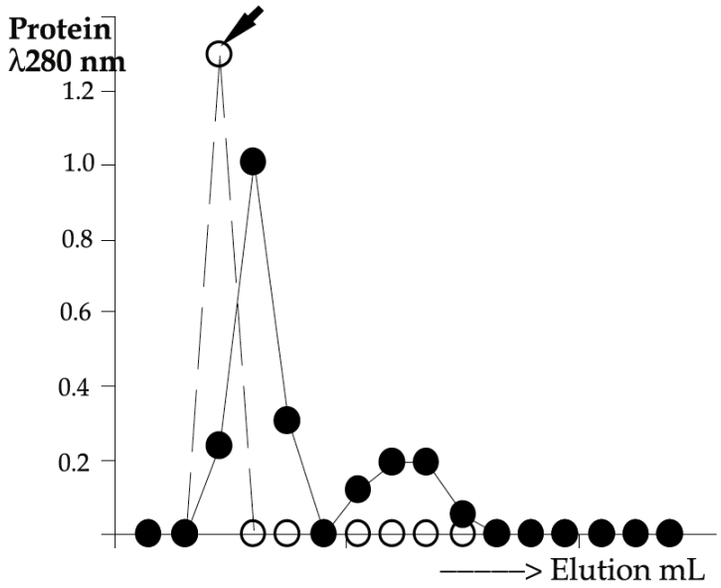

Figure 9. Separation of the f39 and f10 using a Sephadex G50 SEC column (5). The open circle represents absorption profile of AMY (54 kDa) whilst the closed circle is f39 and f10. The arrow marks the peak of AMY.

Further, AMY was pre-treated under various conditions that resulted in denatured and partially denatured enzymes prior to proteolysis. Similar experiments were also carried out using a chemically modified AMY (33). The results were employed to assess the domain organization and assignment of AMY as well as to predict the precise location of trypsin cleavage and the nature of the catalytic domain. These results are being reported elsewhere (33).

7. Conclusion

Amylolytic enzymes from *S. fibuligera* R64 (AMY and GLL1) were successfully separated and identified using chromatography as the key tool. The two enzymes have a different protein surface hydrophobicity profile and their fragmentation profiles provided undisputed proof for their separation. Their assignment was confirmed by the analysis of the products from enzymatic hydrolysis. Further, the domain organization and functioning of AMY has been explored, which led to the structural study of AMY in the absence of its structure. Thereby, this chapter demonstrates how the results from the chromatographic analysis of AMY and GLL1 are complementary to the structural study of the enzymes.

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Abbreviations

AEX, anion exchange chromatography; AMY, α-amylase from *S. fibuligera* R64; CD, cyclodextrin; CEX, cation exchange chromatography; DEAE, diethyl amino ethyl; DNA, deoxyribose nucleic acid; EDTA, ethylene diamine tetra acetate; FPLC, fast protein liquid chromatography; GLA, glucoamylase from *S. fibuligera* KZ; GLL1, glucoamylase from *S. fibuligera* R64; GLU, glucoamylase from *S. fibuligera* HUT7212; HCA, hydrophobic cluster analysis; HIC, hydrophobic interaction chromatography; LC, liquid chromatography; PDB, Protein Data Bank; PGK, phosphoglycerate kinase; RP-HPLC, reversed-phase high performance liquid chromatography; SDS PAGE, sodium dodecyl-sulfate polyacrylamide gel electrophoresis; SEC, size-exclusion chromatography; TFA, trifluoroacetic acid; TLC, thin layer chromatography; TPCK, tosyl phenylalanyl chloromethyl ketone.

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