Amylase modifications during production and application

Amylases are a family of enzymes which catalyse the hydrolysis of a-D-(1,4) glucosidic linkages in starch and related compounds. Amylases are some of the most important industrial enzymes and they are used in a number of different industries one of these being the detergent industry. The commercial amylases are produced by fermentations of genetically modified bacteria, where the native gene has been manipulated to code for an enzyme with improved performance characteristics. Despite the genome only contains copies of the same amylase gene, multiple different amylase forms varying in pI value can be identified when analysing the final enzyme product, hereby indicating that the protein is modified.

The purpose of the present Ph.D. project is to identify differences between the multiple forms of amylase derived from the same gene and determine if or how these various modifications affect the activity and wash performance. The focus of the present project is on amylases from Bacillus species used in the detergent industry. The thesis begins with an introduction to Bacillus amylases, modifications which could cause the multiple forms varying in pI value and mass spectrometry (MS) methods used for the characterisation of these modifications. This is followed by a chapter concerning the methods used, followed by a chapter discussing the results obtained during the project.

Studies of the multiple forms of amylases revealed that the multiple forms occur during the fermentation, where all the forms were observed already early on in the process. Characterisation of the wash performance of the multiple forms from three different Bacillus amylases showed tendencies of decreasing wash performance with decreasing pI values. A more thorough characterisation of pools of Bacillus licheniformis amylase (BLA) varying in pI revealed that the pools containing the forms with the lowest pI values had slightly lower catalytic efficiency on maltoheptaose and amylose. Identification of the modifications responsible for the multiple forms of BLA was performed by tandem mass spectrometry (MS/MS). One of the modifications suspected to be responsible for the multiple forms, i.e. glycations, can be difficult to identify by the fragmentation methods normally used in MS/MS, such as higher-energy C-trap dissociation (HCD) as they tend to cleave of the modification. Therefore, HCD was compared with the soft fragmentations method electron transfer dissociation (ETD), which should be better at identifying these modifications because it leaves the modifications intact.

Both methods were able to identify glycation sites on a model protein, though ETD identified more sites and provided higher sequence coverage for the glycated peptides.

MS/MS analysis by HCD and ETD of the multiple forms of BLA identified deamidations, glycations and carbamylations as the modifications responsible for the forms varying in pI. The modified sites were mainly located on the surface of BLA with a few deamidations in the area around the active site. The modifications were in some cases observed on amino acids involved in stability, activity and substrate binding. Therefore, some of the multiple forms of BLA will most likely differ in stability, activity and performance due to these modifications.

Finally the influence of these modified sites was evaluated by characterisation of BLA variants with single site specific mutations. The BLA variants with mutations mimicking deamidations in the active site and in the substrate binding cleft were found to influence the kinetic parameters (kcat and KM) in different ways. The wash performance was also influenced by these mutations though these effects were mainly positive. Modified Lys and Arg residues suspected to be involved in substrate binding of longer substrates or secondary binding sites were evaluated by site specific mutations to amino acids with neutral side chains of approximately the same size. Hereby, evaluating the effect of removing possible carbohydrate interaction sites. This did not have any effect on the kinetic parameters but it generally resulted in increased wash performances.