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X-Ray Analysis of Yeast Glucoamylases Prepared by Gene Engineering Techniques

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In recent years there has been considerable interest in glucoamylase [exo-1,4 α-D-glucosidase, 1,4-α-D-glucan glucohydrolase, EC 3 2 1 3] because of its industrial importance Glucoamylases from various microbial sources (namely from *Aspergillus* sp , *Rhizopus* sp , *Saccharomycopsis fibuligera*) are used extensively in industry in the production of glucose Unfortunately, wild type glucoamylases are not suited ideally to their industrial roles, having pH of optimum catalysis and thermostability that are too low In order to

engineer an enzyme with superior properties however, one requires at the very least a thorough understanding of the relationship of the enzyme's structure to its function

The yeast, *Saccharomycopsis fibuligera,* produces glucoamylase as an extracellular glycoprotein with 492 amino acids and a molecular weight of the fully glycosylated form of 62 kDa N-glycosidic carbohydrate moieties linked to asparagine make up about 7 kDa and results in multiple isoforms due to glycosylation heterogeneity (Gašperík *et al* 1991) Several years ago we tried to prepare crystals of glucoamylase from the original producer *S fibuligera,* as well as of that expressed in *Saccharomyces cerevisiae* (Gašperík and Hostinová 1993), but we were not successful Probably it was caused by glycosylation microheterogenity of the enzyme For that reason we decided to crystallise a non-glycosylated form of glucoamylase prepared as a recombinant protein expressed in *Escherichia coli* Proteins produced in this way are non-glycosylated because *E coli* does not have the machinery to synthesise and attach the oligosaccharides to proteins

For production of the recombinant enzymes, encoded by the GLA1 and GLU1 genes, we used pET expression system Unfortunately, we found out, that the most of the expressed glucoamylase was present in aggregates, so called inclusion bodies, whereas very low activity was measured in soluble fraction We tried to suppress this undesirable aggregation process by using lower temperature during expression and lower concentration of IPTG The yields of active soluble enzyme increased approximately 10 times, but amount of the aggregated form of enzyme was still very high

Therefore, we decided to use another strategy We worked out the procedure for isolation of the enzymatically active glucoamylases from the inclusion bodies The procedure is based on solubilisation of inclusion bodies in urea and follow-up renaturation of the denatured enzyme The cardinal step of the renaturation process was a high dilution of the denatured enzyme and a fast removal of urea by a reciprocal dialysis in an artificial kidney Finally, contaminating proteins from the renatured glucoamylase were removed by column chromatography with yields of the recombinant glucoamylases about 30 mg and 10 mg, from one litre media, for Gla1 and Glu1 enzymes, respectively With this yields we could start our crystallisation experiments

To prepare crystals of the recombinant glucoamylases we used vapour diffusion technique We started our crystallisation experiments with the Gla1 glucoamylase In initial screening the crystals of glucoamylase Gla1 were produced in the form of needles The needles, however, weren't large enough for data collection Their longest dimension was only about 0 02 mm In next experiments we prepared larger crystals These crystals grew as thin plates, but the problem was that they were highly twinned Similar crystals were also obtained during space mission We tried a lot of crystallisation conditions, but we didn't succeed to get suitable crystals Finally we decided to use microseeding technique and this method appeared as the best method for producing large, morphologically single crystals Crystals diffracted to the resolution 1 5 Å, but they turned out to be fragile and not suitable for data collection

In case of the recombinant glucoamylase Glu1 we were more successful We managed to prepare crystals of a very good quality and crystals of this glucoamylase Glu1 were used for structure determination (Solovicová *et al* 1997)

The structure of the *S fibuligera* glucoamylase was solved by molecular replacement The structure was refined to 1 7 Å with R factor of 14 6 % The overall fold of enzyme is an (a/a) barrel The enzyme molecule is composed from 14 alpha-helices, 12 of them make up an a/a barrel There are no b-sheets 6 inner helices are each other connected through six peripheral alpha helices The remaining two extra short helices are out of a/a barrel structure The putative active site lies in a pocket, at the narrower end of the core

The structure of glucoamylase from *S fibuligera* represents the first crystal structure of yeast glucoamylase Until now only the structure of catalytically active proteolytic fragment of glucoamylase from fungus *A awamori* was solved Despite of the low sequence identity, about 32 %, between these two enzymes, the structures are very similar (Ševčík *et al* 1998)

Significant electron density was observed in the cave of the barrel From its size it was clear that it belongs to a low molecular weight substance From its shape we deduced that it belonged to a Tris molecule It was unexpected, because Tris was not used in crystallisation buffers Tris buffer was, however, used during renaturation and purification of the recombinant glucoamylase Our next experiments proved that Tris had inhibitory effect on glucoamylase activity, which was comparable with a strong glucoamylase inhibitor, acarbose We deduce that the Tris molecule is localised in the active site of the enzyme

Nowadays, we have prepared, for crystallographic studies, the structure of the glucoamylase Glu1 in complex with acarbose

Our future work will be oriented on the preparation of mutants from *S fibuligera* glucoamylases, on their detailed kinetic characterisation in comparison to the wild type enzymes Gla1 and Glu1 and on measurements of physicochemical parameters and comparison with related glucoamylases, specially with *A awamori* glucoamylase The more detailed knowledge on the structure/function relationship of yeast glucoamylases can be used for development of a protein engineered glucoamylase for industrial application

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