Evidence for a polysaccharide-binding domain in *Hormoconis resinae* glucoamylase P: effects of its proteolytic removal on substrate specificity and inhibition by β -cyclodextrin

Richard Fagerström

Tel: +358 0 1332892. Fax: +358 0 1333346.

Research Laboratories Alko
Ltd, POB 350, SF-00101
Helsinki, Finland

The hydrolysis of soluble starch, raw starch and pullulan with recombinant glucoamylase P from *Hormoconis resinae* was competitively inhibited by β -cyclodextrin with apparent K_i values of 190 μ M, 13 μ M and 1.4 μ M, respectively. Inhibition of dextran hydrolysis was partial: a maximum inhibition of 22% was achieved with a dextran concentration of $0.3 \times K_m$ and up to 4 mM β -cyclodextrin. Hydrolysis of short oligosaccharides was not inhibited by β -cyclodextrin at levels up to 20 mM. The enzyme bound to raw starch at pH 4.3 and 4 °C with an association constant of $3.4 \times 10^5 \text{ M}^{-1}$. Sequence alignment studies showed raw-starch-binding consensus amino acids in the C-terminal part of glucoamylase P. Partial hydrolysis with papain resulted in degradation of deglycosylated glucoamylase P into three fragments of 53, 51 and 14 kDa, respectively, as estimated by SDS-PAGE. The amino-terminal sequences of the 51 and 53 kDa fragments were identical with that of native glucoamylase P. The amino terminus of the 14 kDa fragment (Ser-Ser-X-Gln-Val-Ser-), corresponded to the sequence starting at residue 474 of intact glucoamylase P. Kinetic measurements of truncated glucoamylase P showed changes in the K_m values of larger polysaccharides, but no changes in k_{cat} values compared to the intact enzyme. It was concluded that glucoamylase P contains a catalytic core domain and a raw-starch-binding domain involved in inhibition of polysaccharide hydrolysis by β -cyclodextrin.

Keywords: glucoamylase P, *Hormoconis resinae*, polysaccharide-binding domain, starch digestion, enzyme–substrate interaction

INTRODUCTION

The binding and hydrolysis of raw starch by glucoamylases (1,4- α -D-glucan glucohydrolase, EC 3.2.1.3) have been previously studied in detail with *Aspergillus* (Dalmia & Nikolov, 1991; Medda *et al.*, 1982), *Rhizopus* (Takahashi *et al.*, 1985) and *Endomycopsis fibuligera* (Ueda & Saha, 1983). The results have suggested a structural organization of the polypeptides into core (catalytic) and tail (raw-starch-binding) domains (Svensson *et al.*, 1986), as has also been proposed for cellulases (van Tilbeurgh *et al.*, 1986).

The raw-starch-binding domain of *Rhizopus* glucoamylase

has been found to be located in the N-terminus (Takahashi *et al.*, 1985) and that of *Aspergillus* glucoamylase in the C-terminal part of the polypeptide (Svensson *et al.*, 1986). The absence of this domain in one form of native *A. niger* glucoamylase (G2) does not seem to change its kinetic properties towards smaller oligosaccharides compared to the other native form (G1) containing this domain (Meagher *et al.*, 1989). The same results have been obtained by deleting this domain from *Aspergillus* sp. K-27 (Abe *et al.*, 1990) and *A. niger* glucoamylase G1 (Stoffer *et al.*, 1993) by limited proteolysis.

Cyclodextrins have been reported to inhibit many carbohydrate hydrolysing enzymes, such as α -amylase (Hayashida *et al.*, 1990; Kobayashi *et al.*, 1988), β -amylase (Thoma & Koshland, 1960), pullulanase (Marshall, 1973) and some glucoamylases (Savel'ev *et al.*, 1989; Monma *et al.*, 1989; Fukuda *et al.*, 1992). The binding of β -

Abbreviations: DP, degree of polymerization; glucoamylase P_c, 'core' fragment of glucoamylase P; glucoamylase P_t, 'tail' fragment.

cyclodextrin by the isolated raw-starch-binding domain of *Aspergillus* glucoamylase has also been studied (Belshaw & Williamson, 1993).

The high debranching (high activity towards 1,6- α -glycosidic bonds) glucoamylase P from *Hormoconis resinae* (McCleary & Anderson, 1980; Fagerström *et al.*, 1990; Fagerström, 1991) has been cloned and expressed in *Trichoderma reesei* (Joutsjoki & Torkkeli, 1992; Joutsjoki *et al.*, 1993). The recombinant glucoamylase P has been shown to retain the properties of the wild-type enzyme (Fagerström, 1994). The amino acid sequence of this enzyme is similar (47.7% identity) to that of *Aspergillus* glucoamylase. The long highly O-glycosylated threonine/serine-rich region (TS region) proposed to separate the raw-starch-binding domain from the catalytic core of *Aspergillus* glucoamylase G1 seems to be absent in glucoamylase P (Vainio *et al.*, 1993).

None of the glucoamylases, cellulases and other carbohydrate hydrolases shown to contain a separate binding domain have been crystallized intact. Only the core structures have been crystallized (Aleshin *et al.*, 1992; Rouvinen *et al.*, 1990). In the case of cellobiohydrolase II, the structure of the tail part has been obtained by NMR studies (Kraulis *et al.*, 1989). Thus, there is no clear view of whether and how the core and tail interplay during hydrolysis of large carbohydrate substrates.

METHODS

Enzyme preparation. Recombinant *Hormoconis resinae* glucoamylase P was produced in *Trichoderma reesei* (ALKO2743; Joutsjoki *et al.*, 1993) and purified as described by Fagerström (1994). Fraction GAP-A (molecular mass 70 kDa) was used in inhibition and corn-starch-binding experiments. In papain-digestion experiments, a deglycosylated sample was used. The deglycosylation was performed by incubating 3 mg glucoamylase P with 0.2 IU endoglycosidase F (New England BioLabs) in 20 mM sodium acetate, pH 5.5, containing 1 mM PMSF (Sigma) and 10 μ g pepstatin A (Sigma) ml⁻¹ for 23 h at 37 °C. The pH of this digest was elevated by adding 0.1 vol. 0.5 M potassium phosphate, pH 7.5, before further deglycosylation with 0.4 IU of N-glycosidase F (New England BioLabs) for 18 h at 37 °C after addition of fresh PMSF (to 1 mM) and pepstatin A (to 10 μ g ml⁻¹). The deglycosylated sample was purified on a Superose 12 (Pharmacia) gel-exclusion column (1 $\times$ 30 cm) equilibrated with 20 mM sodium acetate buffer, pH 5.7, containing 150 mM NaCl and run at 25 ml h⁻¹. Absorbance at 280 nm was followed. The concentration of protein was estimated spectrophotometrically at 205 nm by the method of Scopes (1974) using an $A_{1\text{cm}}^{1\%}$ value of 307. All enzyme preparations were stored at +4 °C or at -20 °C with only one thawing, because some loss in specific activity was observed after repeated freezings and thawings.

Substrates. Zulkowsky starch (Merck) was used as soluble starch and tested for the presence of shorter oligosaccharides by thin-layer chromatography on silica TLC plates (Merck, silica gel 60) as described by Hansen (1975) with the modification introduced by Melasniemi (1987). Corn starch (raw starch,

Merck) was washed three times prior to use with 50 mM sodium acetate buffer, pH 4.3. Sedimentation between the washings was by centrifugation for 5 min at 2800 g. Pullulan (average degree of polymerization, DP, about 1200; Cattlay, 1979) and the series of maltose to maltoheptaose (over 90% pure) were used as obtained from Sigma. Dextran T-10 (DP about 60) was from Pharmacia. The series of isomaltose to isomaltoheptaose (over 90% pure) was from Biocarb.

Activity measurements and estimations of kinetic constants.

Initial rates were obtained by incubating substrate and enzyme in 50 mM sodium acetate, pH 4.3, containing 150 μ g ovalbumin ml⁻¹ (Sigma, grade V) at 30 °C, as described by Fagerström (1991), unless stated otherwise. Released glucose was measured by using a glucose-dehydrogenase-based assay (Merck, Granulotest 100). One unit of activity is defined as 1 μ mol glucose released min⁻¹. Incubation times of 0 to 30 min were used for pullulan, soluble starch, maltotriose to maltoheptaose, and isomaltotriose to isomaltoheptaose. For isomaltose and maltose, incubation times of 0 to 80 min were used, and in the case of dextran T-10 and corn starch incubations, up to at least 120 min. Inhibition studies with corn starch were performed in 50 mM sodium acetate buffer, pH 4.3, omitting ovalbumin from the reaction mixture. Steady-state rates at incubation times between 60 and 180 min (see, for example, Fig. 3) were used. Preliminary results obtained by shaking (250 r.p.m.) the mixture during incubation showed no difference as compared to samples without shaking. Thus, for convenience, all incubations were performed statically with mixing only before samples were taken. The apparent kinetic constants were estimated by using a computer program based on the method introduced by Cornish-Bowden (1977) to fit the steady-state rate equations. The type of inhibition was estimated from Lineweaver-Burk plots. To obtain apparent K_i values, Dixon plots were used, as well as calculations from the Lineweaver-Burk plots.

Estimation of the association constant of glucoamylase P to corn starch. Binding of 3.2 μ M glucoamylase P to 100 mg corn starch in 1 ml 50 mM sodium acetate, pH 4.3, was followed by incubating the sample under mixing (250 r.p.m., horizontal) at +4 °C. At defined time points samples of 60 μ l were withdrawn and centrifuged (+4 °C) at 6700 g for 3 min. The supernatant was immediately placed in an ice-water bath. In order to estimate the amount of bound enzyme (B) the activities of these supernatants (free enzyme, F) were determined as described above with soluble starch as substrate. In order to determine the total amount of enzyme (T), a parallel experiment without corn starch was performed. The amount of bound enzyme (B) was calculated from $B = T - F$.

Estimation of the association constant (K_a) was performed by measuring the amount of free and total enzyme as above, except that the amount of enzyme used was varied between 1.7 and 8.93 μ M and an equilibration time of 60 min was used. Each tube contained 100 mg corn starch in 1 ml 50 mM sodium acetate, pH 4.3. A Langmuir type of isotherm was chosen (Takahashi *et al.*, 1985). By plotting $1/B$ (bound enzyme) against $1/F$ (free enzyme) an association constant between glucoamylase P and corn starch could be estimated from the slope of the line according to equation (1) (from Takahashi *et al.*, 1985). The line was fitted by using the same direct linear plot program as was used for kinetic measurements (see above).

$$1/B = 1/(B_{\max} \times K \times F) + 1/B_{\max} \quad (1)$$

Production and purification of glucoamylase P papain fragments. A sample of 1.4 mg deglycosylated glucoamylase P was incubated with 50 μ g papain (Sigma) in 50 mM sodium acetate

buffer, pH 5.7, at 37 °C. Samples were taken at defined time points and assayed for activity using soluble starch or pullulan as substrate as described above. After 6.5 h incubation, the digest was applied on a Superose 12 column as described above for the purification of deglycosylated glucoamylase P. Both peak fractions obtained were re-chromatographed on the Superose 12 column. Binding of purified glucoamylase P and its fragments to raw starch was tested by incubating 3 nmol of the corresponding protein with or without 100 mg corn starch for 1 h at 4 °C in 50 mM sodium acetate, pH 4.3. After centrifugation for 5 min at 6700 *g*, binding was detected by running the samples on polyacrylamide slab gels (see below).

SDS-PAGE. Polyacrylamide slab gels (6–16 %) containing 0.1 % SDS were stained with Coomassie Brilliant Blue as described by Laemmli (1970).

Amino-terminal sequencing. This was performed by degrading the samples in a gas-pulsed liquid-phase sequencer (Kalkinen & Tilgmann, 1988). The released PTH-amino acids were analysed on-line by using narrow-bore reverse-phase HPLC.

RESULTS

Inhibition of glucoamylase P

Thin-layer chromatography of Zulkowsky starch showed only minor amounts of oligosaccharides with a DP of 7, and no smaller ones (data not shown). Thus, Zulkowsky starch was taken as a high-DP soluble starch.

Preliminary results of α -, β - and γ -cyclodextrin inhibiting the hydrolysis of pullulan by glucoamylase P are shown in Table 1. They show that β -cyclodextrin was the strongest inhibitor. Inhibition studies were continued with β -cyclodextrin.

Oligosaccharides ranging from maltose to maltoheptaose, and from isomaltose to isomaltoheptaose, were tested at

$5 \times K_m$ (values from Fagerström, 1991) with 20 mM β -cyclodextrin for inhibition. No inhibition could be detected (data not shown).

Inhibition of pullulan hydrolysis by β -cyclodextrin was most pronounced, giving an apparent K_i of $1.4 \pm 0.3 \mu\text{M}$ (Table 2). The type of inhibition was competitive (data not shown).

Hydrolysis of soluble starch and corn starch was also inhibited competitively, with apparent K_i values of $190 \pm 56 \mu\text{M}$ and $13 \pm 3 \mu\text{M}$ respectively for β -cyclodextrin (Table 2). The high K_m for corn starch and dextran T-10 should be looked on with caution, since only up to about $3 \times K_m$ values of substrate (100 mg ml^{-1}) were used. High substrate concentrations were avoided because of possible mass transfer problems (Miranda *et al.*, 1991). Inhibition by β -cyclodextrin of the hydrolysis of dextran T-10 appeared to be partial. As seen in Table 3, the inhibition reached maxima of 22 % and 8 %, respectively, at substrate concentrations of about $0.3 \times K_m$ and about $2.5 \times K_m$ when up to 4 mM β -cyclodextrin was used.

Binding of glucoamylase P to corn starch

Binding of glucoamylase P to corn starch at +4 °C reached equilibrium in 10 min when 3.2 μg enzyme and 100 mg corn starch were used (data not shown). The association constant of glucoamylase P to corn starch was estimated from the plot of $1/F$ against $1/B$ (where F and B are free and bound enzyme respectively), using an equilibration time of 60 min. By extrapolation, a maximum binding of roughly 6 nmol glucoamylase P could be calculated, corresponding to about 420 μg glucoamylase P per 100 mg corn starch. From the slope of this plot a K_a value of $3.4 \times 10^3 \text{ M}^{-1}$ for glucoamylase P binding to corn starch could be obtained.

Activity of fragments of glucoamylase P

Partial hydrolysis of deglycosylated glucoamylase P with papain was followed by SDS-PAGE (Fig. 1a) and by activity measurements using soluble starch and pullulan as substrate (Table 4). The initial hydrolysis of the native deglycosylated 63 kDa glucoamylase P was rapid, and seemed to reach a plateau in about 3 to 6 h. During prolonged incubation (up to 30 h) the 53 kDa band that appeared was further digested to a 51 kDa band. The smallest product of the digestion had a molecular mass of 14 kDa as estimated from SDS-PAGE. The initial velocity of soluble starch digestion slightly increased (by about 8 %) during incubation, whereas that for pullulan decreased by about 85 %. After 6.5 h incubation the large fragment, glucoamylase P_c (the 'core'), was separated by gel-exclusion chromatography from the small fragment, glucoamylase P_t (the 'tail'). The amino-terminal sequence of glucoamylase P_c obtained was identical with that of native glucoamylase P (Asp-Leu-Ser-Ser-Phe-Ile-Ala-; Fagerström *et al.*, 1990) and corresponded to the amount of protein used in sequencing. No other sequences could

Table 1. Inhibition of pullulan hydrolysis by glucoamylase P with different cyclodextrins

Activity was measured as described in Methods, except that the incubation temperature was 37 °C. The amount of pullulan used was 5 mg ml^{-1} and amount of enzyme 1.4 $\mu\text{g ml}^{-1}$.

Inhibitor	Concentration (mg ml^{-1})	Inhibition (%)
α -Cyclodextrin	2	12
	0.2	0
β -Cyclodextrin	2	87
	0.2	73
γ -Cyclodextrin	2	67
	0.2	24

Table 2. Apparent K_m and K_i values obtained for glucoamylase P with different substrates and β -cyclodextrin as inhibitor

The estimates of K_i are expressed as means $\pm$ range from the values obtained from Lineweaver-Burk and Dixon plots.

Substrate	K_m (mg ml ⁻¹)	K_i (μ M)
Soluble starch	0.06	190 $\pm$ 56
Pullulan	0.11	1.4 $\pm$ 0.3
Dextran T-10	29.3	...
Corn starch	31.0	13 $\pm$ 3

*No apparent K_i could be obtained for dextran T-10 (see Results and Table 3).

be detected. The amino-terminal sequence of glucoamylase P_t was Ser-Ser-x-Gln-Val-Ser-Ile-Thr-Phe-Asn-, corresponding to residues 474 to 483 of the intact glucoamylase P sequence (Vainio *et al.*, 1993).

Binding of glucoamylase P_t , glucoamylase P_c and glucoamylase P to corn starch was tested by incubating purified samples with corn starch in the ratio 3 nmol per 100 mg (half saturation of glucoamylase P). Binding of gluco-

Table 3. Inhibition by β -cyclodextrin of dextran T-10 hydrolysis by glucoamylase P

Activity was measured in 50 mM sodium acetate, pH 4.3, at 30 °C, as described in Methods. The amount of enzyme used was 1.75 μ g ml⁻¹.

Concn of β -cyclodextrin (μ M)	Concn of dextran T-10 (mg ml ⁻¹)	Inhibition (%)
0.1	75	4
0.1	10	6
2	75	6
2	10	14
10	75	8
10	10	22
4000	75	8
4000	10	21

amylase P and P_t to corn starch was apparent, but no binding of glucoamylase P_c could be detected (Fig. 1b).

Estimations of the apparent K_m and k_{cat} values of deglycosylated glucoamylase P, glucoamylase P_c and a

Fig. 1. SDS-PAGE analysis of glucoamylase P digested with papain. (a) Deglycosylated glucoamylase P (1.4 mg) was incubated with 25 μ g papain ml⁻¹ in 50 mM sodium acetate, pH 5.7, at 37 °C for the following times. Lane 2, 0 min; lane 3, 10 min; lane 4, 30 min; lane 5, 60 min; lane 6, 3.5 h; lane 7, 6.5 h; lane 8, 14 h; lane 9, 24 h; lane 10, 30 h; lane 11, deglycosylated glucoamylase P kept at 37 °C for 30 h. Lanes 13 and 14, purified glucoamylase P_c and glucoamylase P_t , respectively. Lanes 1, 12 and 15, molecular mass markers. (b) Purified glucoamylase P, P_c and P_t were incubated at 4 °C with or without corn starch in a ratio of 3 nmol per 100 mg each, as described in Methods. Lanes 2, 4 and 6, glucoamylase P, P_c and P_t , respectively, without corn starch; lanes 3, 5 and 7, the respective samples in the presence of corn starch; lane 1, molecular mass markers.

Table 4. Relative activity of glucoamylase P towards 5 mg soluble starch ml⁻¹ or pullulan during digestion by papain

Deglycosylated glucoamylase P (1.4 mg ml⁻¹) and papain (50 µg ml⁻¹) were incubated at 37 °C in 50 mM sodium acetate, pH 5.7. Samples at defined time points were assayed for activity using either soluble starch or pullulan as substrate (5 mg ml⁻¹ each) as described in Methods.

Incubation time (h)	Relative activity (%)	
	Soluble starch	Pullulan
0	100	100
0.17	109	85
3.5	109	17
6.5	109	16
10.0	106	14
14.0	102	14

mixture of glucoamylase P_c and glucoamylase P_t in a 1:2 molar ratio with different substrates are shown in Table 5. No apparent changes in K_m and k_{cat} could be seen for the malto- and isomalto-oligosaccharides tested. There was a tenfold increase in K_m for soluble starch when glucoamylase P_c was used, without any change in k_{cat} . Glucoamylase P_c acting on pullulan showed a 400-fold increase in K_m , but no change in k_{cat} . Changes of the

kinetic constants upon adding glucoamylase P_t to glucoamylase P_c were within the SD values of the measurements.

The apparent K_m and k_{cat} for glucoamylase P_c towards corn starch could not be estimated because of the low activity.

The progress curves (Fig. 2) of glucoamylase P acting on corn starch showed an initial 'burst' followed by a linear steady-state rate. Possible inactivation of glucoamylase P in reaction mixtures was studied by measuring the activity of the mixtures towards soluble starch after incubation in the presence or absence of 20 or 100 mg corn starch ml⁻¹ (Fig. 2). In all three cases about 40% of the original activity disappeared during 3 h. In reaction mixtures containing 100 mg soluble starch ml⁻¹ no inactivation could be detected, and the progress curves were linear (not shown).

Addition of fresh substrate (100 mg ml⁻¹) during corn starch hydrolysis (100 mg corn starch ml⁻¹ and 50 µg glucoamylase P ml⁻¹) resulted in a new 'burst' followed by a new steady-state (Fig. 3). This addition diluted the reaction mixture by 1:1. Roughly the same steady state was achieved by diluting the original mixture by 1:1 with buffer. Addition of more enzyme to 50 mg corn starch ml⁻¹ caused a smaller 'burst' followed by a new steady-state rate.

The plots of the linear steady-state rate against the amount of glucoamylase P were linear at enzyme to substrate ratios above 0.25 µg per mg corn starch (Fig. 4). A much smaller linear steady-state rate was obtained with glucoamylase P_c hydrolysing corn starch.

Table 5. Kinetic constants of papain-digested and undigested glucoamylase P

The enzyme preparations were purified as described in the text and the kinetic constants were estimated with a variety of substrates. Glucoamylase P, deglycosylated undigested glucoamylase P; glucoamylase P_c, purified core domain of glucoamylase P; glucoamylase P_c + P_t, 1 mol glucoamylase P_c and 2 mol purified raw-starch-binding domain, glucoamylase P_t. Estimates are means ± range of at least two independent experiments, except for isomaltoheptaose. The K_m of pullulan with glucoamylase P_c and the combination of glucoamylase P_c and P_t could not be measured accurately, since the maximum amount of substrate used was only 2–3 × K_m .

Substrate	Glucoamylase P		Glucoamylase P _c		Glucoamylase P _c + P _t	
	K_m	k_{cat} *	K_m	k_{cat} *	K_m	k_{cat} *
Soluble starch†	0.06 ± 0.01	30 ± 1	0.67 ± 0.14	29 ± 1	0.78 ± 0.20	30 ± 1
Pullulan†	0.10 ± 0.02	18 ± 2	~ 40	18 ± 1	~ 50	17 ± 1
Maltose‡	1.8 ± 0.1	5.8 ± 0.1	2.1 ± 0.1	6.1 ± 0.2	2.0 ± 0.2	5.6 ± 0.2
Maltoheptaose‡	0.26 ± 0.03	38 ± 2	0.25 ± 0.04	37 ± 2	0.20 ± 0.02	40 ± 2
Isomaltose‡	21 ± 1	2.8 ± 0.3	22 ± 2	3.0 ± 0.2	23 ± 1	2.8 ± 0.1
Isomaltoheptaose‡	4.5	11	4.8	12	4.6	12
Corn starch†	37 ± 5	0.62 ± 0.07	ND	ND	ND	ND

ND, Not determined.

* k_{cat} expressed as µmol min⁻¹ mg⁻¹.

† K_m expressed as mg ml⁻¹.

‡ K_m expressed as mM.

Fig. 2. Progress curves for glucoamylase P acting on corn starch. Samples of $50 \mu\text{g}$ glucoamylase P ml^{-1} were incubated with 20 mg (○) and 100 mg (●) corn starch ml^{-1} respectively at 30°C in 50 mM sodium acetate, $\text{pH } 4.3$. Released glucose was measured at defined time points as described in Methods. The error bars represent deviations of an average of at least three independent experiments. Activity towards soluble starch was measured during incubations with: ▽, 20 mg corn starch ml^{-1} ; □, 100 mg corn starch ml^{-1} and ×, without corn starch. Relative activities were calculated.

Fig. 3. Effect of dilution, addition of substrate and enzyme on the linear steady-state rate. A sample of $50 \mu\text{g}$ glucoamylase P ml^{-1} was incubated with 100 mg corn starch ml^{-1} at 30°C in 50 mM sodium acetate, $\text{pH } 4.3$ (●). After 90 min , part of the incubation mixture was withdrawn and diluted (1:1) with buffer (△). To another part was added 100 mg corn starch ml^{-1} (×), resulting in a dilution of the mixture by 1:1. An additional sample of $50 \mu\text{g}$ ml^{-1} was incubated with 50 mg corn starch ml^{-1} (○). After 90 min , part of the mixture ($500 \mu\text{l}$) was withdrawn, and a concentrated sample of glucoamylase P ($50 \mu\text{g}$ in $10 \mu\text{l}$) was added (▽).

DISCUSSION

Belshaw & Williamson (1990) reported an association constant of $3.8 \times 10^5 \text{ M}^{-1}$ between *A. niger* glucoamylase (form G1) and corn starch, whereas Dalmia & Nikolov (1991) found a constant one order of magnitude higher. Takahashi *et al.* (1985) reported a value of $1.2 \times 10^5 \text{ M}^{-1}$ for *Rhizopus* glucoamylase (G1). The association constant of $3.4 \times 10^5 \text{ M}^{-1}$ obtained in this study for glucoamylase P from *Hormoconis resinae* is in broad agreement with those values. The binding capacity of corn starch for glucoamylase P was about $4 \text{ mg protein (g corn starch)}^{-1}$, close to the values reported for *A. niger* glucoamylase G1 [$1.6\text{--}4.3 \text{ mg protein (g corn starch)}^{-1}$; Dalmia & Nikolov, 1991]. This suggests that glucoamylase P from *Hormoconis resinae* also contains a 'high affinity' raw-starch-binding domain. Removal of this domain did not alter the specificity towards small malto and isomalto oligo-

saccharides (Table 5). Apparently the raw-starch-binding domain is not involved in the debranching activity of glucoamylase P.

Studies on the separated binding domain of *A. niger* glucoamylase (Belshaw & Williamson, 1991) have revealed that it binds $2 \text{ mol } \beta\text{-cyclodextrin (mol protein)}^{-1}$. Further, this domain seems to contain at least two subsites, of which one (s1) binds malto-oligosaccharides, raw starch, dextrans and $\beta\text{-cyclodextrin}$ (Belshaw & Williamson, 1993). The other subsite (s2) would bind the proposed transition state analogue (glucono-1,5-lactone) and also contributes to dextran binding. The low K_i ($1.4 \pm 0.3 \mu\text{M}$) for $\beta\text{-cyclodextrin}$ inhibiting pullulan hydrolysis by glucoamylase P is close to the K_d value of $\beta\text{-cyclodextrin}$ from the raw-starch-binding domain of *A. niger* glucoamylase ($1.68 \mu\text{M}$ for the second ligand; Belshaw & Williamson, 1991). Consider-

Fig. 4. Effect of enzyme concentration on corn starch digestion. Steady-state rates of different amounts of glucoamylase P hydrolysing 20 mg (×) and 100 mg (●) corn starch ml^{-1} in 50 mM sodium acetate, pH 4.3, at 30 °C were measured. The same measurements were performed with glucoamylase P_c (○ and △, respectively).

ing the high homology between these glucoamylases (except for the TS region), this suggests that the K_i reflects binding of β -cyclodextrin to an s1 site in glucoamylase P, and so that pullulan also binds to this site in the raw-starch-binding domain. The big increase of K_m (400-fold) upon removal of the tail of glucoamylase P supports this proposal. This increase in K_m explains the apparent loss of activity towards pullulan during partial proteolysis with papain (Table 4), because the substrate concentration used changes from about $50 \times K_m$ to about $0.1 \times K_m$.

A possible explanation for the partial inhibition observed with dextran T-10 as substrate could be that subsite s1 becomes fully occupied with β -cyclodextrin, compelling dextran T-10 to bind only to a subsite s2 of the raw-starch-binding domain, still enabling hydrolysis to occur.

The apparent K_i of β -cyclodextrin for glucoamylase P acting on corn starch ($13 \pm 3 \mu\text{M}$) and the type of inhibition are similar to those reported for the isolated raw-starch-binding domain of *A. niger* glucoamylase (competitive, apparent K_i $11.0 \pm 1.9 \mu\text{M}$; Bellshaw & Williamson, 1991).

Hydrolysis of shorter malto- and isomalto-oligosaccharides (DP 2–7) with glucoamylase P was not inhibited by β -cyclodextrin. This has also been shown for *Aspergillus* glucoamylase acting on malto-oligosaccharides (Fukuda *et al.*, 1992). With glucoamylase P, β -cyclodextrin inhibits the hydrolysis of soluble starch with a K_i one

order of magnitude lower than reported for *Chlarax paradoxax* (Monma *et al.*, 1989) and *Aspergillus awamori* (Fukuda *et al.*, 1992) glucoamylases.

The rapid deceleration during the first 20 min of corn starch hydrolysis to a steady-state rate did not seem to be solely a result of inactivation of the enzyme (Fig. 2). Inactivation of about 15% in 20 min would not be expected to slow down the rate to one-fifth. Addition of fresh substrate caused a new burst, suggesting that the deceleration was not due to product inhibition. Addition of fresh enzyme to mixtures of low enzyme/substrate ratios caused a new burst, but smaller than the first. These results suggest that the initial rate (the burst) is mainly due to some minor but reactive component(s) of the corn starch, such as insoluble structures or residues of soluble components, whereas the steady-state rate reflects hydrolysis of large corn starch granules.

The hydrolysis rate of corn starch with glucoamylase P_c was much smaller than with glucoamylase P (Fig. 4). Small amounts (about 1–3%) of intact glucoamylase P as an impurity in glucoamylase P_c preparations could however result in about the same degree of hydrolysis as obtained. Thus, no definite proof of glucoamylase P_c digesting corn starch could be obtained.

Incubations of 1 mg deglycosylated glucoamylase P ml^{-1} with 20 mg corn starch ml^{-1} at 30 °C for 2.5 d resulted in about 10% hydrolysis as estimated from the theoretical value (unpublished data). This is in agreement with the results obtained with *A. awamori* var. *kawachi* glucoamylase G1 hydrolysing corn starch (Hayashida *et al.*, 1989). This slow hydrolysis of corn starch has to be taken into account when industrial applications involving raw starch hydrolysis are planned. An important effect of the raw-starch-binding domain from an industrial point of view is that it lowers the K_m of large soluble polysaccharide substrates, resulting in efficient hydrolysis at low concentrations.

Sequence alignment of the raw-starch-binding and linker region of glucoamylase P with other glucoamylases is shown in Fig. 5. There are nine consensus sequence amino acids present in glucoamylase P out of 11 proposed for different starch-degrading enzymes (Svensson *et al.*, 1989). The absence of the highly glycosylated TS region in glucoamylase P is apparent.

The role of the TS region in hydrolysis of insoluble substrates has led to conflicting interpretations. Hayashida *et al.* (1989) suggested that this TS region was important in raw starch digestion by glucoamylase G1 from *A. awamori* var. *kawachi*. Later results have suggested a minor effect of this region on binding of the enzyme to raw starch (Williamson *et al.*, 1992). From the results obtained here, it seems that a long TS region is not needed for efficient binding of glucoamylase to raw starch. However its effect on efficient hydrolysis of raw starch may still be of importance.

The apparently short linker region in glucoamylase P may result in less flexibility between the core and tail domain.

```

GP(439) SWGSRNALPPPTTC SA SS T P GIYTPATAAGA (469)
AN(436) ---ETSASSV-G--AAT--IGTY--V-VTSW-SIVATG-TT-T--PT-S (484)
          ↑                ↑
        Minor cleavage siteb Major cleavage siteb

          Major cleavage sitea
          ↓
GP(470) PNVTS SC QVSITFNINATTYYGENLYV (496)
AN(485) GS---TSKTTATASKTSTSTSTSTCTPTA-AV--DLT---T----I-L (533)
cons. T G

GP(497) IGNSSDLGAWNADAYPLSASAYTQDRPLWSAAIPLNAGEVISYQYVRQ (545)
AN(534) V-SI-Q--D-ETS-GIA---DK--SSD---YVTVT-P---SFE-KFI-I (582)
cons. G LG W P W K

GP(546) EDCQDPYIYETVNRILTVP ACGGAAVTTDDAWMGPGVSSGNC* (587)
AN(583) -SD-SVEWESDP--EY---Q---TSTA-VT-T-R* (616)
cons. W N

```

Fig. 5. Sequence alignment of glucoamylase P and *A. niger* glucoamylase G1. The consensus (cons.) sequence of different glucoamylases (Svensson *et al.*, 1989) as well as the linker region (from Stoffer *et al.*, 1993) are aligned. Lines represent identity; empty spaces represent gaps in the alignment. Numbering is according to the native amino acid sequence. GP, glucoamylase P; AN, *A. niger* glucoamylase G1. ^a, cleavage site in glucoamylase P by papain digestion (this paper). ^b, cleavage sites found by subtilisin digestion of *A. niger* glucoamylase G1 (Stoffer *et al.*, 1993).

Trials to crystallize this glucoamylase in an intact form with the tail domain are under way.

ACKNOWLEDGEMENTS

I thank Mr John Denslow for computer programming and Dr John Londesborough for critical reading of the manuscript. Dr Nisse Kalkkinen is also acknowledged for amino acid sequencing and Ms Arja Mikkilinen for art work.

REFERENCES

- Abe, J., Nakajima, K. & Hizukuri, S. (1990). Alteration of the properties of *Aspergillus* sp. K-27 glucoamylase on limited proteolysis with subtilisin. *Carbohydr Res* **203**, 129–138.
- Aleshin, A., Golubev, A., Firsov, L. M. & Honzatko, R. B. (1992). Crystal Structure of glucoamylase from *Aspergillus awamori* var. X100 to 2.2 Å Resolution. *J Biol Chem* **267**, 19291–19298.
- Belshaw, N. J. & Williamson, G. (1990). Production and purification of a granular-starch-binding domain of glucoamylase 1 from *Aspergillus niger*. *FEBS Lett* **269**, 350–353.
- Belshaw, N. J. & Williamson, G. (1991). Interaction of β -cyclodextrin with the granular starch binding domain of glucoamylase. *Biochim Biophys Acta* **1078**, 117–120.
- Belshaw, N. J. & Williamson, G. (1993). Specificity of the binding domain of glucoamylase 1. *Eur J Biochem* **211**, 717–724.
- Cattlay, B. J. (1979). Pullulan synthesis by *Aureobasidium pullulans*. In *Microbial Polysaccharides and Polysaccharases*, pp. 69–84. Edited by R. C. E. Berkeley, G. W. Gooday & D. C. Ellwood. London: Academic Press.

- Cornish-Bowden, A. (1977). An automatic method for deriving steady-state rate equations. *Biochem J* **165**, 55–59.
- Dalmia, B. K. & Nikolov, Z. L. (1991). Characterization of glucoamylase adsorption to raw starch. *Enzyme Microb Technol* **13**, 982–990.
- Fagerström, R. (1991). Subsite mapping of *Hormoconis resiniae* glucoamylases and their inhibition by gluconolactone. *J Gen Microbiol* **137**, 1001–1008.
- Fagerström, R. (1994). Purification and specificity of recombinant *Hormoconis resiniae* glucoamylase P and endogenous glucoamylase from *Trichoderma reesei*. *Enzyme Microb Technol* **16**, 36–42.
- Fagerström, R., Vainio, A., Suoranta, K., Pakula, T., Kalkkinen, N. & Torkkeli, H. (1990). Comparison of two glucoamylases from *Hormoconis resiniae*. *J Gen Microbiol* **136**, 913–920.
- Fukuda, K., Teramoto, Y., Goto, M., Sakamoto, J., Mitsui, S. & Hayashida, S. (1992). Specific inhibition by cyclodextrins of raw starch digestion by fungal glucoamylase. *Biosci Biotechnol Biochem* **56**, 556–559.
- Hansen, S. A. (1975). Thin-layer chromatographic method for identification of oligosaccharides in starch hydrolyzates. *J Chromatogr* **105**, 338–390.
- Hayashida, S., Nakahara, K., Kanlayakrit, W., Hara, T. & Teramoto, Y. (1989). Characteristics and function of raw-starch-affinity site on *Aspergillus awamori* var. *kawachi* glucoamylase I molecule. *Agric Biol Chem* **53**, 143–149.
- Hayashida, S., Teramoto, Y., Inoue, T. & Mitsui, S. (1990). Occurrence of an affinity site apart from the active site on the raw-starch-binding but non-raw-starch-adsorbable *Bacillus subtilis* 65 α -amylase. *Appl Environ Microbiol* **56**, 2584–2586.
- Joutsjoki, V. V. & Torkkeli, T. K. (1992). Glucoamylase P gene of *Hormoconis resiniae*: Molecular cloning, sequencing and introduction into *Trichoderma reesei*. *FEMS Microbiol Lett* **99**, 237–244.
- Joutsjoki, V., Torkkeli, T. & Nevalainen, H. (1993). Transformation of *Trichoderma reesei* with the *Hormoconis resiniae* glucoamylase P (*gamP*) gene: production of a heterologous glucoamylase by *Trichoderma reesei*. *Curr Genet* **24**, 223–228.
- Kalkkinen, N. & Tilgmann, C. (1988). A gas-pulsed-phase sequencer constructed from a Beckman 890D instrument by using Applied Biosystems delivery and cartridge blocks. *J Protein Chem* **7**, 242–243.
- Kobayashi, M., Takagi, S., Matsuda, K. & Ichishima, E. (1988). Inhibition of α -amylase and phosphorylases by cyclodextrin-dialdehyde. *Agric Biol Chem* **52**, 2703–2708.
- Kraulis, P. J., Clore, G. M., Nilges, M., Jones, T. A., Pettersson, G., Knowles, J. & Gronenborn, A. M. (1989). Determination of the three-dimensional structure of the C-terminal domain of cellobiohydrolase I from *Trichoderma reesei*. A study using nuclear magnetic resonance and hybrid distance geometry–dynamical simulated annealing. *Biochemistry* **28**, 7241–7257.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Marshall, J. J. (1973). Inhibition of pullulanase by Sharding dextrins. *FEBS Lett* **37**, 269–273.
- McCleary, B. V. & Anderson, M. A. (1980). Hydrolysis of α -D-glucans and α -D-glucosaccharides by *Cladosporium resiniae* glucoamylases. *Carbohydr Res* **86**, 77–96.
- Meagher, M. M., Nikolov, Z. L. & Reilly, P. J. (1989). Subsite mapping of *Aspergillus niger* glucoamylase I and II with malto- and isomaltooligosaccharides. *Biotechnol Bioeng* **34**, 681–688.
- Medda, S., Saha, B. C. & Ueda, S. (1982). Raw starch adsorption and elution behavior of glucoamylase I of black *Aspergillus*. *J Fermentation Technol* **60**, 261–264.

- Melasniemi, H. (1987).** Effect of carbon source on production of thermostable α -amylase, pullulanase and α -glucosidase by *Clostridium thermohydrosulfuricum*. *J Gen Microbiol* **133**, 883–890.
- Miranda, M., Murado, M. A., Sanroman, A. & Lema, J. M. (1991).** Mass transfer control of enzymatic hydrolysis of polysaccharides by glucoamylase. *Enzyme Microb Technol* **13**, 142–147.
- Monma, M., Yamamoto, Y. & Kainuma, K. (1989).** Subsite structure of *Cblara paradoxa* glucoamylase and interaction of the glucoamylase with cyclodextrins. *Agric Biol Chem* **53**, 1503–1508.
- Rouvinen, J., Bergfors, T., Teeri, T., Knowles, J. K. C. & Jones, T. A. (1990).** The three dimensional structure of cellobiohydrolase II from *Trichoderma reesei*. *Science* **249**, 380–386.
- Scopes, R. K. (1974).** Measurement of protein by spectrophotometry at 205 nm. *Anal Biochem* **59**, 277–282.
- Savel'ev, A. N., Sergeev, V. R. & Firsov, L. M. (1989).** Analysis of an additional substrate-binding site of glucoamylase I. The effect of cyclodextrin on glucoamylase-catalyzed reactions. *Biokhimiya* **54**, 1725–1731.
- Stoffer, B., Frandsen, T. P., Busk, P. K., Schneider, P., Svendsen, I. & Svensson, B. (1993).** Production, purification and characterization of the catalytic domain of glucoamylase from *Aspergillus niger*. *Biochem J* **292**, 197–202.
- Svensson, B., Larsen, K. & Gunnarson, A. (1986).** Characterisation of a glucoamylase G2 from *Aspergillus niger*. *Eur J Biochem* **154**, 497–502.
- Svensson, B., Jespersen, H., Sierks, M. R. & MacGregor, E. A. (1989).** Sequence homology between putative raw-starch binding domains from different starch-degrading enzymes. *Biochem J* **264**, 309–311.
- Takahashi, T., Kato, K., Ikegami, Y. & Irie, M. (1985).** Different behavior towards raw starch of three forms of glucoamylase from *Rhizopus* sp. *J Biochem* **98**, 663–671.
- Thoma, J. A. & Koshland, D. E. (1960).** Competitive inhibition by substrate during enzyme action. Evidence for the induced-fit theory. *J Am Chem Soc* **82**, 3329–3333.
- Ueda, S. & Saha, B. C. (1983).** Behaviour of *Endomycopsis fibuligera* glucoamylase towards raw starch. *Enzyme Microb Technol* **5**, 196–198.
- Vainio, A. E. I., Torkkeli, H. T., Tuusa, T., Aho, S. A., Fagerström, B. R. & Korhola, M. P. (1993).** Cloning and expression of *Hormoconis resinae* glucoamylase P cDNA in *Saccharomyces cerevisiae*. *Curr Genet* **24**, 38–44.
- van Tilbeurgh, H., Tomme, P., Claeysens, M., Bhikhabhai, R. & Petterson, G. (1986).** Limited proteolysis of the cellobiohydrolase I from *Trichoderma reesei*. Separation of functional domains. *FEBS Lett* **204**, 223–227.
- Williamson, G., Belshaw, N. J. & Williamson, M. P. (1992).** O-glycosylation in *Aspergillus* glucoamylase. Conformation and role in binding. *Biochem J* **282**, 423–428.

Received 23 December 1993; revised 23 March 1994; accepted 6 April 1994.