Protein heterogeneity of spinach pullulanase results from the coexistence of interconvertible isomeric forms of the monomeric enzyme

Anette HENKER*, Ilka SCHINDLER*, Andreas RENZ† and Erwin BECK*¹

*Lehrstuhl für Pflanzenphysiologie, Universität Bayreuth, Universitätsstrasse 30, D 95440 Bayreuth, Germany, and †Max-Planck-Institut für molekulare Pflanzenphysiologie, Karl-Liebknecht-Strasse 25, D14476 Golm, Germany

Purified pullulanase (starch-debranching enzyme, R-enzyme, EC 3.2.1.41) from spinach (*Spinacia oleracea* L.) chloroplasts separated into at least seven individual enzymically active proteins (isomers, numbered 1–7) on isoelectric focusing or column chromatofocusing. At their isoelectric points (between pH 4.7 and 5.2) these forms were rather stable. At slightly alkaline pH, each converted into the whole set of isomers. PAGE of the purified enzyme under denaturing or non-denaturing conditions resulted in one protein band. When substrate (amylopectin or pullulan) was included in the gel, the native enzyme as well as any of the individual isomers separated into two (sometimes three) bands ('substrate-induced forms', numbered I–III) with different specific activities, dissociation constants of the enzyme–substrate complexes and activation energies. Each substrate-induced form

produced the whole set of seven isomers on isoelectric focusing. The specific activity of the total enzyme reflected the relative proportions of the substrate-induced forms. To some extent the relative proportions, as determined by crossed immunoelectrophoresis, could be shifted in favour of the more or the less active forms by reduction with dithiothreitol, and gentle oxidation respectively. Activation by dithiothreitol did not alter the mode of action of the enzyme but only increased the velocity of substrate degradation and extended its activity into the pH range of the chloroplast. As a consequence of isomer interconversion, microheterogeneity could serve to regulate pullulanase activity in a biochemical manner that shares some features with allosteric regulation.

INTRODUCTION

Starch-degrading enzymes represent the classical examples of enzyme heterogeneity or multiplicity, i.e. the coexistence of several separable proteins that catalyse apparently the same reaction. The variant forms of such enzymes have been termed isoenzymes or multiple forms and have been compared with the isotopes of an element, since they are subsumed under the same EC number [1]. Regulatory properties, substrate specificities and inter- or intra-cellular compartmentation are the characteristics used to differentiate these multiple forms. Commonly, the term isoenzymes is employed for proteins translated from different mRNAs, whereas multiple forms are understood to result from post-translational protein modification [2]. Purified spinach leaf pullulanase (debranching enzyme, EC 3.2.1.41, frequently termed 'R-enzyme') [3], which debranches soluble starch, like other amylases, shows heterogeneity on electrophoresis in substrate-containing polyacrylamide gels [4] and on isoelectric focusing (IEF) [2]. However, because only one single band is obtained for the spinach pullulanase on SDS/PAGE (molecular mass ≈ 102 kDa) or gel chromatography (molecular mass ≈ 110 kDa), considerable post-translational modification is unlikely to be the reason for the formation of the multiple forms of this protein [4]. From the total amino acid sequence, a molecular mass of 99 kDa is calculated for the mature protein [5], which is released from a precursor containing a chloroplast signal peptide of 65 amino acid residues. As confirmed in the following paper [6], the formation of several coexistent forms is a property of this individual protein. In the present communication, we characterize the individual free and substrate-induced multiple forms of the spinach chloroplast pullulanase, show their interconvertibility and suggest a biochemical consequence of the coexistence of several isomers of the same protein in one cellular compartment.

EXPERIMENTAL

Preparation of spinach chloroplast pullulanase

Routinely, spinach chloroplast pullulanase was purified from a leaf homogenate by $(\text{NH}_4)_2\text{SO}_4$ precipitation, affinity-column chromatography on Sepharose-bound cyclohepta-amylose (β -cyclodextrin) with a gradient of cyclohepta-amylose in the eluent, and removal of β -cyclodextrin by molecular-sieve chromatography on Sephadex G-25. Cyclohepta-amylose is a very potent inhibitor of the enzyme. Recovery is 7–14%, concomitant with a purification of up to 7000-fold. Details of the procedure by which the pure protein is obtained have been described in an earlier report [4], when the enzyme was characterized with respect to kinetic parameters, pH optimum, substrate specificity and activation by thiol reagents. Alternatively, the enzyme can be prepared from a protein extract of isolated spinach chloroplasts, in which case $(\text{NH}_4)_2\text{SO}_4$ precipitation can be omitted. In this case the recovery is 34% and the purification about 400-fold [4]. The purified enzyme can be stored without loss of activity at -20°C for at least 4 weeks.

Enzyme assays

Routinely, enzyme activity was determined in a stop time assay with pullulan (ICN, Cleveland, OH, U.S.A.) as substrate by measuring the liberated reducing groups with the dinitrosalicylic acid reagent [7]. This test has the disadvantage that the efficiency of the reaction depends on the species of the reducing saccharide and thus varies from carbohydrate to carbohydrate; therefore an exact quantification of a mixture of oligosaccharides is not possible. The activities presented in this paper refer to maltotriose as reference, which is the final product of pullulan degradation

Abbreviations used: IEF, isoelectric focusing; DTT, dithiothreitol.

¹ To whom correspondence should be addressed (e-mail erwin.beck@uni-bayreuth.de).

by a pullulanase-type debrancher. The assay mixture consisted of 150 μ l of 100 mM Mes/NaOH buffer, pH 5.8, containing 2 mM NaF and 4.5 mg/ml pullulan, to which was added 75 μ l of protein solution. After 15, 30 or 60 min incubation at 37 °C, 200 μ l of dinitrosalicylic acid reagent was added, and the mixture was allowed to react in a thermobloc at 100 °C for 5 min. After cooling to room temperature, 300 μ l of the sample was placed in a well of a microtitre plate and the absorption was determined with an ELISA reader (EAR 400T; SLT-Labinstruments, Gröding, Austria). In the presence of thiol reagents, this assay is less precise because of a co-operative effect of the reducing groups and the thiol on the dinitrosalicylic acid. In this case an assay with Red Pullulan was used [8], which is not affected by 2-mercaptoethanol, dithiothreitol (DTT) or glutathione. In this assay, 200 μ l of a 2% solution of Red Pullulan (Megazyme, Sydney, Australia) in 500 mM KCl solution was added to 400 μ l of protein solution and incubated for 30 min at 40 °C. The reaction was terminated with 1 ml of 85% ethanol, and undegraded pullulan was removed by centrifugation at 3000 g (10 min at 4 °C). The absorbance of the clear supernatant was read at 518 and/or 543 nm. The assay was calibrated with a standard sample of pullulanase, the activity of which was determined with the dinitrosalicylic acid reagent in the absence of thiol reagent. One unit of pullulanase activity liberates 1 μ mol of reducing groups from pullulan in 1 min.

Automatic sugar analysis

For identification of the degradation pattern of pullulan by the spinach pullulanase, the enzymic reaction was terminated by boiling the reaction mixture for 10 min and dilution with 2 vol. of 0.1 M borate buffer, pH 8. Precipitated protein was removed by centrifugation and 100 μ l of the supernatant was injected into an automatic sugar analyser (Carbohydrate Analyzer LC 2000; Biotronik, Frankfurt, Germany). A Durrum DA-X4-20 column (Dionex) was used from which the mono- and oligo-saccharides were eluted with a step gradient of borate buffer (0.1 M, pH 8.0, to 0.5 M, pH 10.0). For quantitative detection the eluate was allowed to react with Orcine reagent (0.5 g of Orcine in 1 litre of 97% H₂SO₄), and A_{440} was continuously read.

Determination of protein

Protein was routinely measured with the Bio-Rad protein assay (Bio-Rad, Munich, Germany). However, when protein was dissolved in Polybuffer on chromatofocusing, this assay gave erroneous results. In this case, the Biuret reagent (Pierce Chemical Co., Rockford, IL, U.S.A.) was used. BSA was used for calibration.

PAGE

For non-denaturing PAGE, slab gels (10 cm $\times$ 11.5 cm $\times$ 0.1 cm) were prepared which usually contained 0.1% amylopectin or 1% Red Pullulan for activity staining. After electrophoresis (6 h at 4 °C and 200 V), the gel buffer was replaced by 0.1 M sodium acetate buffer, pH 6.0, containing 1 mM DTT or 14 mM 2-mercaptoethanol, and the gels were incubated for 3–4 h at 30 °C. Subsequently, zymograms were produced by staining with I₂/KI (30 g of KI and 13 g of I₂ in 1 litre of water; 1:10 diluted). Debranching enzyme activity was detected by a light blue colour, resulting from the reaction of debranched α -(1 $\rightarrow$ 4)-linked polyglucans with iodine. In the case of Red Pullulan, colourless bands appeared on a red background. Proteins were stained with Coomassie Brilliant Blue R250 (Boehringer Ingelheim Bioproducts, Heidelberg, Germany) or with silver nitrate [9].

SDS/PAGE

Because of the high molecular mass of the R-enzyme, a 10% polyacrylamide gel with a low degree of cross-linking (acrylamide/*N,N*-methylenebisacrylamide 75:1, w/w) was used for SDS/PAGE, which otherwise was performed as described by Laemmli [10]. Protein staining on the gels was as described above.

IEF

IEF was performed as described by Robertson et al. [11] in vertical minigels (Mini-Protean II electrophoresis chamber; BioRad) of 6% acrylamide with ampholytes (pH 4–6) from Serva (Boehringer Ingelheim Bioproducts). The samples were mixed with application buffer (60% glycerol, 4% Servalyt 4–6) at a ratio of 1:1 (v/v). The electrolyte of the cathode was 25 mM NaOH, and that of the anode 20 mM acetic acid. IEF was performed for 1.5 h at 200 V, followed by another 1.5 h at 400 V. After fixation with 30% ethanol, proteins were stained with Coomassie Brilliant Blue or silver nitrate. For activity staining, the indirect method of MacGregor et al. [12] was used: 0.2 mm-thick gels which contained 1% amylopectin or 2% Red Pullulan and 0.1 M sodium acetate buffer were layered on the developed IEF gel and kept there for 60 min. With Red Pullulan, colourless bands were immediately visible after washing with water, whereas the amylopectin-containing gels had to be stained with KI/I₂ to visualize pullulanase activity as light blue bands. Calibration proteins were obtained from Sigma (Munich, Germany): trypsin inhibitor (pI 4.55), egg albumin (pI 4.8), β -lactoglobulin A (from milk, pI 5.13), carbonic anhydrase B (bovine erythrocytes, pI 5.85).

Chromatofocusing

A Mono P HR 5/20 column (20 cm $\times$ 0.5 cm; bed volume 4 ml; Pharmacia, Freiburg, Germany) was used for chromatofocusing. The column was connected to an FPLC® system (LKB, Bromma, Sweden) equipped with a gradient mixer, which provided an almost linear pH gradient from 5.2 to 4.65 in Polybuffer® 74 (Pharmacia). Protein concentration was recorded continuously and fractions of 0.4 ml were taken for determination of the enzyme activity.

Determination of dissociation constants and activation energies

Dissociation constants were determined as described by Gerbrandy [13] and Matsumoto et al. [14], using non-denaturing gel electrophoresis on polyacrylamide gels containing 0, 0.05, 0.1 and 0.15% amylopectin. The runs were performed at 5, 10 and 15 °C. From the mobility of the substrate-induced forms II and III, which decreased with increasing substrate concentration and decreasing temperature, a Lineweaver–Burk plot and an Arrhenius plot were produced. From these plots, K_D and the activation energies were calculated.

Production of antibodies and crossed immunoelectrophoresis

A rabbit was subcutaneously immunized three times with 150 μ g of highly purified protein in periods of 2 weeks. After another 4 weeks, it was boosted with 50 μ g of protein. For injection, pullulanase protein was mixed with Freund's adjuvant (Sigma) at a ratio of 1:1 (v/v). Only for the first immunization was complete adjuvant (i.e. with killed mycobacteria) used. At 2 or 3 weeks after the last booster injection, blood was collected from the ear vein, and, after centrifugation, was either used or stored at –20 °C.

Crossed immunoelectrophoresis was performed by the method of Weeke [15] in 1% agarose gels (Agarose Type I; Sigma) containing 40 mM Tris/HCl buffer, pH 8.6, containing 100 mM glycine and 0.1% Thimerosal. Electrophoresis in the first direction (12 V/cm) was in an amylopectin-containing gel (0.4%). Antiserum (0.6%) was included in the agarose gel for the second direction. The second run was at 2 V/cm overnight. Unprecipitated protein was removed by repeated pressing of the gel and subsequent soaking in 0.15 M NaCl, and, after drying with a fan, the precipitated protein was stained with Serva Blue R250 in 35% ethanol and 10% acetic acid (30 min). For destaining (1–2 min), 50% propan-2-ol was used.

RESULTS

Free and substrate-induced isomeric forms

Pure spinach pullulanase, which produced only one protein band on both PAGE in non-denaturing gels and SDS/PAGE, resolved into up to seven discrete bands (termed 1–7) by IEF which all showed enzyme activity (Figure 1). Isoelectric points between pH 4.7 and 5.2 were determined for the individual forms. In non-denaturing polyacrylamide gels, which contained amylopectin or pullulan as substrate, the enzyme activity of the pullulanase resolved into two, sometimes three, bands (termed I, II and III; Figure 1B), the mobility of which depended on the temperature of the run (Figure 2A), and on the concentration of substrate in the gel (Figure 2B), and thus indicated the formation of the enzyme–substrate complex at the pH of the buffer (pH 8.6). Since the enzyme did not show any activity at this pH (Figure 3), only the dissociation constants and the activation energy of complex-formation could be determined (Table 1). The less mobile form II had a lower activation energy ($28 \text{ kJ} \cdot \text{mol}^{-1}$) than the faster migrating form III ($56 \text{ kJ} \cdot \text{mol}^{-1}$). The least mobile form (form I) exhibited low activity and was only sporadically seen, and therefore could not be investigated in detail. In the absence of substrate, or when glycogen, a non-cleavable polyglucan, was co-polymerized in the gel, or when the inhibitor cyclohepta-amylose was added to the gel buffer, the enzyme,

Figure 1 IEF (A) and non-denaturing PAGE (B) of spinach leaf pullulanase

(A) Separation of isomers of spinach leaf pullulanase by IEF in the absence of substrate. (A1) Silver staining of proteins; (A2) activity staining. The proteins were transferred after IEF to an amylopectin-containing polyacrylamide gel and incubated at pH 6. After 2 h, the gel was stained with iodine. Amylose, produced by the action of pullulanase, stained blue against the purple background colour of the amylopectin–iodine complex. (B) Non-denaturing PAGE of spinach pullulanase on an amylopectin-containing gel. After electrophoresis, the gel was incubated at pH 6 for 3 h and then stained with iodine. The light blue colour shows the activity of the pullulanase.

Figure 2 Dependence of the dissociation constants of the substrate-induced forms II and III of spinach leaf pullulanase on the temperature (A) during PAGE, and the concentration of the amylopectin in the polyacrylamide gel (B)

Closed symbols, form II; open symbols, form III. The temperatures were 5 °C ($\diamond$), 10 °C ($\circ$) and 15 °C ($\triangle$). $R_{f_{\max}}$ represents the relative mobility in amylopectin-free gels.

appearing as a single band, showed high mobility in the polyacrylamide gel [4]. Thus the two (or three) bands produced in the presence of a suitable substrate represent substrate-induced forms, which because of interaction with the immobilized substrate, showed retarded electrophoretic mobility.

Figure 3 Dependence of the activity of the spinach leaf pullulanase on the pH without DTT ($\circ$) and with 10 mM DTT in the reaction mixture ($\bullet$)

Table 1 Characterization of the substrate-induced forms II and III of spinach leaf pullulanase

Parameter	Form II	Form III
Specific activity with pullulan (units/mg of protein at pH 6.0)	56	104
Specific activity with amylopectin (units/mg of protein at pH 6.0)	37.9 ± 19.8	71.6 ± 31.2
Dissociation constants (mg of amylopectin/ml)		
At 5 °C, pH 8.6	0.21	0.44
At 15 °C, pH 8.6	0.31	0.95
Activation energy for formation of the enzyme-amylopectin complex ($\text{kJ} \cdot \text{mol}^{-1}$)	28	56
V_{max} of the total enzyme at pH 6.0 with Pullulan		59
Amylopectin		19

Interconvertibility of the pullulanase isomers

On crossed immunoelectrophoresis with a polyclonal antibody against highly purified spinach pullulanase, and when amylopectin was present in the gel matrix of the first run, a continuous line of up to three peaks without spurs was observed, whereas only one peak resulted in the absence of substrate (Figure 4). This finding suggested a close antigenic relationship between the substrate-induced forms. When any one of the free forms or the substrate-induced forms were electroeluted from the gels and refocused or again subjected to electrophoresis in substrate-containing gel, the whole set of isoforms was obtained (results not shown). Sometimes the original band prevailed, but the others were always present. This behaviour of the protein confirmed the idea of a single protein that coexists as several isomers of different net charge ('charge isomers') and with different affinity for the substrate (substrate-induced forms).

Figure 4 Crossed immunoelectrophoresis of purified spinach leaf pullulanase

(A) Without substrate in the gel; (B) 2% amylopectin was added to the agarose gel for the first run. A polyclonal antibody against the pure enzyme was included in the gel for the second run. Protein was stained with Coomassie Blue.

Figure 5 Chromatofocusing of spinach leaf pullulanase

(A) Chromatofocusing of purified spinach leaf pullulanase on a Mono P/HR column. The numbers 1–7 indicate the individual isomers. (B) Rechromatofocusing of isomer 4 after incubation at pH 8 for 12 h.

Table 2 Characterization of the isomeric forms of spinach leaf pullulanase

The data are given as means $\pm$ S.E.M. $n = 4$ for isomers 4 and 5, $n = 3$ for isomer 7 and $n = 2$ for isomers 3 and 6. n.d., not determined.

Isomer number	Isoelectric point	Protein content (μ g/isomer)	Specific activity (units/mg of protein)	Total activity (units/isomer)
1	5.08–5.16	n.d.	n.d.	0.08
2	5.03–5.07	n.d.	n.d.	0.20
3	4.96–5.01	11.3 ± 4.3	10 ± 0.4	0.22 ± 0.04
4	4.92–4.96	24.8 ± 3.4	138 ± 53	4.2 ± 1.3
5	4.87–4.91	36.7 ± 5.3	211 ± 90	6.7 ± 2.4
6	4.79–4.85	10.9 ± 3.4	36 ± 11	0.82 ± 0.34
7	4.72–4.79	22.6 ± 7.5	114 ± 73	2.8 ± 1.7

Isolation and characterization of the individual isomers

Chromatofocusing of homogeneous and substrate-free spinach pullulanase on a Mono P HR 5/20 column yielded up to seven coincident protein and activity peaks (Figure 5A), just as on IEF. Rechromatofocusing of the individual proteins showed a relatively high preservation of the separated forms if they were maintained at pH 5 close to their isoelectric points. Interconversion of the individual forms was very efficient at alkaline pH, as shown by rechromatofocusing of form 4 after incubation at pH 8 (Tris/HCl) overnight at room temperature (Figure 5B). However, even at pH 8.5, interconversion was not complete with all seven forms, but on the other hand, none of these forms was completely preserved in the alkaline milieu. Isomers 4 and 5 interconverted more readily into the whole set of forms than the others.

The individual forms exhibited different specific activities, as shown in Table 2. Form 5, representing a little more than one-third of the protein, was by far the most active one, and forms 4 and 7 on the one hand, and 3 and 6 on the other had similar but significantly lower specific activities. The amount of protein in forms 1 and 2 was so small that specific activities could not be determined.

Change of the contribution of the individual forms to the overall activity

The substrate-induced forms II and III were electroeluted from a non-denaturing polyacrylamide gel and the specific activities were determined after adjustment of the pH to 6.0. Only 50% of the activity applied to the gel could be recovered. The specific activities of forms II and III were significantly different: 37.9 ± 19.8 units/mg of protein for form II and 71.6 ± 31.2 units/mg of protein for form III; thus form III was about twice as active as form II (Table 1). Determination of the specific activity of the free forms necessarily led to the conversion into substrate-induced forms. Different specific activities of the individual free forms suggest preferential formation of one of the substrate-induced forms. Hence, by changing the shares of the individual forms, the overall activity of the R-enzyme should be modulatable. The proportions of the individual substrate-induced forms in the protein solution could be estimated from the peak sizes obtained on crossed immunoelectrophoresis (Figures 6A and 6B). A shift of the shares in favour of form III was accomplished by treatment with DTT. Concomitantly, the specific activity increased (Table 3). Figure 6(C) shows that this shift was pH-dependent and most effective between pH 7 and 8. However, a complete shift to only one of the forms could not be

Figure 6 Analysis by crossed immunoelectrophoresis of the formation of forms II and III

(A) In the absence of DTT; (B) in the presence of 10 mM DTT. Conditions were as described in Figure 4. (C) Dependence of the proportions of forms II and III on the pH. Open symbols, controls; closed symbols, in the presence of 10 mM DTT.

Table 3 Change in activity of spinach leaf pullulanase caused by reduction and oxidation

Identical amounts of enzyme, the activity of which was set at 100%, were used in the assays. Preceding oxidation or reduction was for 30 min each.

Reducing agent	Effect (% of control)	Oxidizing agent	Effect (% of control)	Re-activation with:	Effect (% of control)
Control	100	—	—	—	—
10 mM DTT	130	20 mM Diamide	60	25 mM DTT	110
20 mM DTT	155	40 mM Diamide	50	45 mM DTT	120
—	—	10 mM Diamide	80	25 mM DTT	150
20 mM DTT	170	40 mM H ₂ O ₂	55	50 mM DTT	120
—	—	10 mM H ₂ O ₂	10	25 mM DTT	120

achieved with the SH reagent DTT. On the other hand, mild oxidation of the protein with diamide or diluted H₂O₂ resulted in a significant decrease in the overall specific activity concomitant with a shift in favour of form II. As shown in Table 3, the effects of reduction and oxidation were reversible, suggesting that the

Figure 7 Lineweaver-Burk plot of spinach leaf pullulanase without, and in the presence of, 20 mM DTT

forms differ in their redox state. Treatment with DTT did not affect the apparent K_m , but only doubled V_{max} (Figure 7). Simultaneously, the range of enzyme activity was significantly extended into the alkaline milieu (Figure 3). However, the mode of action did not change, as indicated by a time course of the appearance of the degradation products maltotriose, (maltotriose)₂ and (maltotriose)₃ (Figure 8).

DISCUSSION

Coexistence of differently charged isomers

Direct evidence is presented in the following paper [6] that spinach pullulanase is the product of one gene which, apart from cleavage of a chloroplast signal peptide, is not altered by post-translational modification. The mature enzyme is a monomeric protein of about 100 kDa. The coexistence of several separable and, at their isoelectric points, stable isoforms is therefore a feature of the protein itself. The interconvertibility of the individual forms described in this paper is another strong argument for identity of their amino acid sequences. The phenomenon, which is contradictory to the idea of only one

thermodynamically favourable structure of a protein in a given milieu [17], appears to be a special case of protein polymorphism and will be termed protein microheterogeneity. So far, a few enzymes have been reported to show protein polymorphism in this manner. However, the biological significance of this phenomenon has not been investigated. One of these enzymes is rabbit muscle adenylate kinase, three isomers of which with apparently different molecular masses (22–29 kDa) have been separated by gel chromatography [18]. These isomers have been termed conformers and have been interpreted as more open or more closed molecular shapes. Since the individual forms of spinach pullulanase can be separated and identified by their isoelectric points, the term ‘charge isomers of the monomeric protein’ may be more appropriate than conformers. The chemical reason for the coexistence of these isomers is not yet clear. Because they cannot be separated by gel chromatography, their molecular shapes may be more similar than those of rabbit muscle adenylate kinase. Since the protein contains nine cysteine residues [5] and because reduction and mild oxidation altered the shares of the individual isomers (Table 3), thiol-disulphide interconversion may be involved in the microheterogeneity. The situation may alternatively be described by the coexistence of several low-enthalpy states of the protein, which, depending on the milieu, are populated to different degrees by the protein molecules. In the absence of substrate, up to seven of these states could be separated by IEF or chromatofocusing but not by non-denaturing PAGE. The differences in charge at the pH of the gel buffer (pH 8.6) are presumably too small for electrophoretic separation. Even the isoelectric points are very close, ranging from pH 4.72 to 5.16 (Table 2).

Coexistence and formation of several substrate-induced forms

With a suitable substrate immobilized in the gel, up to three pullulanase activities could be resolved electrophoretically. Since the electrophoretic mobilities of these forms were lower than that of the enzyme in a gel containing non-cleavable glycogen instead of amylopectin, the separation of the three activities must mainly be due to differing strong interactions of the enzyme molecules with the substrate. Obviously this interaction produces up to three forms with different dissociation constants, which, because of the undefined size of the polyglucan, can only be calculated in terms of per gram concentration of amylopectin or pullulan in

Figure 8 Time kinetics of the degradation of pullulan by spinach leaf pullulanase without (●) and in the presence of (○) 10 mM DTT

Maltotriose, maltotriosehexose and maltotriosenonose were separated and quantified in an automatic sugar analyser (Carbohydrate Analyzer LC 2000) as described by Hansen and Beck [16]. (A), 2 h; (B), 7 h; (C), 20 h. Abbreviation: P, polymer.

the gel (Table 1). Presumably, these dissociation constants do not reflect different binding sites for the substrate, which have been reported for the glycogen debrancher system from rabbit skeletal muscle, the amylo-1,6-glucosidase and the 4- α -glucanotransferase (EC 3.2.1.33 and EC 2.4.1.25) [19]. Rather, the pullulanase isomers are interpreted as being substrate-induced isomers exhibiting the same binding site in slightly varied molecular structures. It should be noted that the dissociation constants only refer to the formation of the enzyme-substrate complexes, because the enzyme was inactive at the pH of the gel buffer. However, after readjustment of the pH to 6, activity could be restored. On the other hand, separation of the isomers by IEF was independent of the presence or absence of substrate because the enzyme activity, as a result of substrate binding, only started in the pH range between 4.7 and 5.2 (Figure 3). Interestingly, the more active form III had a higher dissociation constant than the less active form II. Such a finding is reasonable, if binding of the substrates takes place not only via the α -1 $\rightarrow$ 6-linked, but also through adjacent α -1 $\rightarrow$ 4-bound glucose moieties, as it is known to occur for other glucan-degrading endohydrolases [19,20]. Dissociation of the debranched glucan chains from the binding site(s) again requires activation energy, and therefore a higher tendency to dissociate may increase, rather than decrease, the catalytic activity of the active site.

If the addition of substrate reduces the number of isomers from seven to three, the question of a correspondence of one of the isolated isomers to one of the substrate-induced isomers arises. For several reasons such a correspondence could not be demonstrated. (i) The substrate-induced forms could only be separated by PAGE, and extraction of the proteins from the gels required a slightly alkaline milieu, which inevitably resulted in interconversion of the forms. In addition, such a procedure resulted in loss of enzyme activity, and the substantial statistical errors shown in Tables 1 and 2 may be due to both effects. (ii) It cannot be completely ruled out that addition of substrate, and formation of the substrate-induced forms *per se*, leads to some interconversion of the protein forms, resulting in various combinations of the substrate-induced forms. Again, a considerable statistical error in the specific activities of the isomers (Table 2) suggests such a possibility. Nevertheless, the pairs of forms 3 and 6, and 4 and 7, respectively each show comparable specific activities, suggesting the formation of similar combinations of substrate-induced forms. Form 5, however, should preferentially produce form III, as indicated by the by-far highest specific activity. Conversion of the isomers into form III requires about twice as much activation energy as conversion into form II; an obvious explanation is that the conformation of form 5 (which is frequently the form with the highest share) allows the molecule to convert more readily into form III than do the conformations of the other six forms.

It is an open question why seven discrete isomers of the substrate-free protein produce only three substrate-induced forms. However, it is conceivable that the ability of the protein to coexist in several isoforms is a prerequisite for the formation of more than one form on binding of substrate. The clue for the physiological interpretation of the coexistence of several substrate-induced forms is the different specific activities of these forms. Hence, the overall specific activity of the operative spinach pullulanase results from the shares of the individual isomers or the populations of the above mentioned low-enthalpy states. Activation by thiols (Figure 7) increases the share of form III and isomer 4 (I. Schindler, unpublished work). Activation by treatment with reductants of inactive debranching enzymes (pullulan-

ases) of cereal seeds has been reported for rice [21], oat and other cereals (reviewed by Nakamura [22]). Activation of these enzymes was observed on germination and could also be achieved by limited proteolysis with papain. Although the pullulanase from germinating rice seeds shares some properties with that from spinach chloroplasts (such as molecular mass, average isoelectric point, the range of specific activity) [21,22], microheterogeneity has not been reported. Thus the two enzymes may differ in a major aspect, and activation by thiols of the two enzymes may not reflect an identical reaction. As shown in Figure 3, the major effect of DTT on spinach pullulanase is a substantial, but asymmetric, broadening of the pH range at which the enzyme shows activity. In the non-reduced form, the enzyme shows hardly any activity at the physiological pH of the chloroplast stroma (pH 7–8) [23]. Thus the increase in activity at the optimal pH (5.9) is less important than the preservation of activity at pH 7–7.5. This idea corresponds to a favoured formation of the more active form III at this pH (Figure 6C). As a consequence, the activity of the thiol-treated presumably reduced enzyme is under the control of the diurnal chloroplastic pH oscillations [23], whereas the oxidized enzyme shows hardly any activity in this range. Furthermore, the phenomenon of microheterogeneity appears to serve to regulate the pullulanase activity in a biochemical manner which, in depending on different forms of the polypeptide, shares some features with allosteric regulation. However, it is less stringent than the typical allosteric regulation, since a complete interconversion of one isomer into the others could not be achieved and since, in the absence of substrate, there is an unusually high number of coexisting isomers.

We thank Professor Franz Xaver Schmid, Department of Biochemistry, University of Bayreuth, for helpful discussions and valuable suggestions. The work was partly sponsored by the Deutsche Forschungsgemeinschaft in the scope of the Graduiertenkolleg 'Biosynthese der Proteine und Regulation ihrer Aktivität'.

REFERENCES

- Moss, D. W. (1982) Isoenzymes, Chapman and Hall, New York
- Beck, E. and Ziegler, P. (1989) Annu. Rev. Plant Physiol. Plant Mol. Biol. **40**, 95–117
- Hobson, P. N., Whelan, S. and Peat, S. (1951) J. Chem. Sci. **1951**, 1451–1459
- Ludwig, I., Ziegler, P. and Beck, E. (1984) Plant Physiol. **74**, 856–861
- Renz, A., Schmid, R., Kossmann, J. and Beck, E. (1995) Plant Physiol. **108**, 1342
- Renz, A., Schikora, S., Schmid, R., Kossmann, J. and Beck, E. (1998) Biochem. J. **330**, 937–945
- Bernfeld, P. (1955) Methods Enzymol. **1**, 149–158
- Serre, L. and Laurière, C. (1990) Anal. Biochem. **186**, 312–315
- Heukeshoven, J. and Dernick, R. (1985) Electrophoresis **6**, 103–112
- Laemmli, U. K. (1970) Nature (London) **227**, 680–685
- Robertson, E. F., Dannelly, K., Malloy, P. J. and Reeves, H. C. (1987) Anal. Biochem. **167**, 290–294
- MacGregor, A. W., Thompson, R. G. and Meredith, W. O. S. (1974) J. Inst. Brew. **80**, 181–185
- Gerbrandy, S. J. (1974) Biochim. Biophys. Acta **370**, 410–418
- Matsumoto, A., Nakajima, T. and Matsuda, K. (1990) J. Biochem. (Tokyo) **107**, 123–126
- Weeke, B. (1973) Scand. J. Immunol. **2**, 47–56
- Hansen, J. and Beck, E. (1994) Trees **8**, 172–182
- Creighton, T. E. (1990) Biochem. J. **270**, 1–16
- Russell, P. J., Chinn, E., Williams, A., David-Dimarino, C., Taulane, J. P. and Lopez, R. (1990) J. Biol. Chem. **265**, 11804–11809
- Takrama, J. and Madsen, N. B. (1988) Biochemistry **27**, 3308–3314
- MacGregor, E. A. (1985) Carbohydr. Res. **142**, 223–236
- Iwaki, K. and Fuwa, H. (1981) Agric. Biol. Chem. **45**, 2683–2688
- Nakamura, Y. (1996) Plant Sci. **121**, 1–18
- Werdan, K., Heldt, H. W. and Milovancev, M. (1975) Biochim. Biophys. Acta **396**, 276–292