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PURIFICATION AND CHARACTERIZATION OF α-AMYLASE FROM POPLAR LEAVES

WOLFGANG WITT and JÖRG J. SAUTER

Botanisches Institut der Universität Kiel, Olshausenstr. 40, D-24098 Kiel, Germany

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Key Word Index—*Populus* × *canadensis*; Salicaceae; poplar; enzyme purification; starch degradation; α-amylase; thioredoxin.

Abstract—Endoamylase (EC 3.2.1.1) from mature poplar leaves (*Populus* × *canadensis* Moench "*robusta*") was purified 17 700-fold over the crude protein extract to a specific activity of 166 μmol min^{-1} mg^{-1} by $(NH_4)_2SO_4$-precipitation, treatment with anion exchange resins, affinity chromatography on a starch grain column, and gel filtration. The purified enzyme was classified as α-amylase by its substrate specificity and by comparison with poplar wood α-amylase. It migrates on SDS-PAGE gels as a single band (M_r 44 000), but it shows electrophoretic polymorphism as detected by activity staining on native PAGE gels containing amylopectin. The purified α-amylase is reversibly inactivated by oxidation in the absence of reducing agents and by chelation of divalent cations. The activity was restored by reductants and metal ions, a combination of Ca^{2+}, DTT, and thioredoxin being most effective. The amylase was stable at 30° over several hr in the presence of DTT and thioredoxin whereas 2-mercaptoethanol could stabilize the activity only at 4°. The treatment with divalent cations and reductants also changed the native PAGE banding pattern and resulted in a shift of the pH optimum to a higher value. It is concluded that the electrophoretic polymorphism is caused by the different affinity to the immobilized amylopectin of the enzyme forms associated with the individual bands. Some of these effects were also observed with the main endoamylase from poplar wood.

INTRODUCTION

Investigations of the enzymatic mechanism of starch degradation in several plant tissues led to the assumption that α-amylases play a major role in the attack on starch granules *in vivo* [1, 2]. A variety of α-amylases, mostly from microbial, mammalian and cereal sources, are well characterized. These enzymes belong to a large family of Ca^{2+}-proteins, which share several structural features [3,4].

Endoamylases from higher plants other than cereals were less well characterized although leaf and seed α-amylases were purified from several species. Partly contradictory results were reported regarding the ability to degrade native starch granules and the requirements for optimal activity. Endoamylases from most sources readily attacked starch granules, but pea α-amylase showed hardly any activity with this substrate [5]. Spinach [6] and barley leaf [7] α-amylases could be treated with EDTA without inactivation whereas the enzyme usually requires Ca^{2+} for stability and activity. Furthermore, purified endoamylases from many sources were separated into multiple forms by electrophoretic procedures. This electrophoretic polymorphism was shown to arise by different post-translational modifications [8–10] or by loss of Ca^{2+} during the separation [5]. These examples demonstrate that the properties of this enzyme from different plant sources may vary considerably in spite of the close structural relationship.

Recently, the main groups of amylolytic enzymes from poplar wood were completely separated and partially purified [11]. Combination experiments indicated that the poplar α-amylase may play a major role in starch degradation in this material [12]. The partially purified endoamylase also showed multiple bands in native PAGE [11], and seasonal variations of the banding pattern were closely correlated with phases of starch degradation [13, 14].

In the present investigation, a procedure for the complete purification of the main endoamylase from poplar was developed with the aim to understand the electrophoretic polymorphism and to analyse some enzymatic properties without interference by isoenzymes and other starch-degrading enzymes. The reversible inactivation by oxidation of sulfhydryls and chelation of divalent cations was especially investigated.

RESULTS

Purification of α-amylase

Poplar leaves contained a rather low endoamylase activity compared with other plant enzyme sources. Less

Table 1. Summary of the purification of endoamylase from poplar leaves

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units mg^{-1})	Purification	Yield (%)
Crude Extract	4130	38.7	0.0094	1	100
DEAE Cellulose	2420	36.2	0.0149	1.6	94
$(NH_4)_2SO_4$	817	20.5	0.0250	2.7	53
Q Sepharose	314	18.0	0.0574	6.1	47
Starch binding	0.371	5.77	15.6	1660	15
Gel filtration/ 2nd Q Sepharose	0.028	4.64	166	17700	11

The activity was tested at pH 6.5 with β-limit dextrin from potato as substrate. A unit is defined as μmol reducing power min^{-1}.

than 1% of the specific activity in extracts from pea shoots [15] and pea cotyledons [16] was found in the crude poplar leaf extract (Table 1). An extremely high purification was therefore necessary to obtain an electrophoretically homogeneous enzyme preparation.

Endoamylase from poplar does not bind to anion exchange resins in media of slightly alkaline pH [11]. This property was used together with ammonium sulfate precipitation as a rapid initial cleaning procedure (Table 1). The most effective purification step was affinity chromatography with starch grains from potato (Fig. 1). The major part of the amylase was retarded on a column packed with this material. This activity was eluted by prolonged washing of the column with buffer, whereas the bulk protein eluted in a single peak near the void volume. The amylase activity in this peak was discarded to obtain about 270-fold purification. The starch grain affinity procedure was adapted from earlier reports on the purification of starch-degrading enzymes [16–18]. The final purification was achieved by gel filtration and a second passage through Q-Sepharose. The typical result of a complete purification is summarized in Table 1. Endoamylase was 17 700-fold purified over the crude extract to a specific activity of 166 μmol min^{-1} mg^{-1} protein with a yield of 11%. The recovery may have been underestimated because the substrate, β-limit dextrin, is also attacked by other amylases from poplar [11]. In total, four complete purifications with very similar results were carried out.

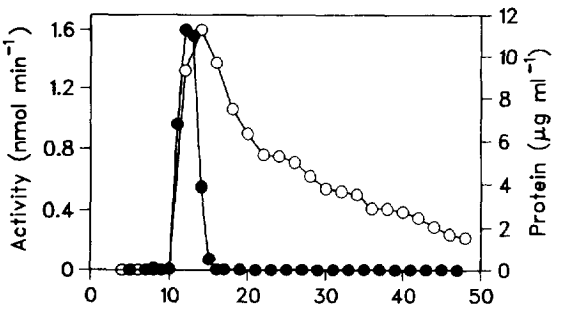

Fig. 1. Starch grain affinity chromatography of poplar leaf α-amylase. The protein concentration (●) and the amylase activity (○) using starch granules from corn as substrates was determined in the fractions.

M_r and electrophoretic polymorphism

SDS-PAGE of the purified α-amylase revealed a single band running at M_r 44000 (results not shown). Five major bands – labelled LA1–LA5 in Fig. 2 – were consistently visualized by native PAGE and activity staining of the same samples. In some cases, a minor band running between LA1 and LA2 and two or three bands of high electrophoretic mobility were detected; the latter bands may result from partial inactivation due to the loss of divalent cations (see below). The major bands were also found in crude leaf extracts (not shown). Partially purified poplar wood endoamylase exhibited the same pattern of five bands [11], which was also present in crude enzyme extracts from this material (Figs 3 and 4).

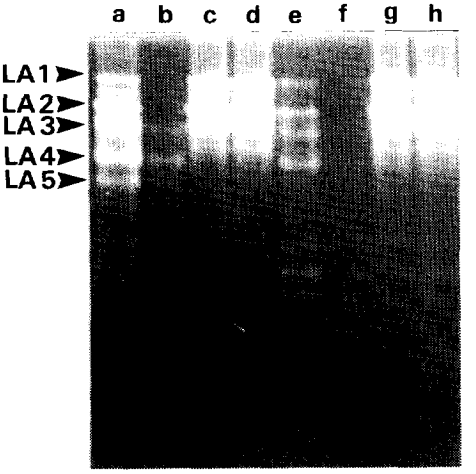

Fig. 2. Native PAGE of α-amylase samples after treatment with reducing agents. Purified α-amylase from poplar leaves was incubated with or without reductants for 20 hr at 4° (lanes a–d) or for 1 hr at 30° (lanes e–h) as in the experiment of Fig. 6. Control desalted in buffer C (lanes a and e); samples desalted in the same solution but without 2-mercaptoethanol (lanes b and f); samples without 2-mercaptoethanol treated with DTT (lanes c and g) or with DTT and TR (lanes d and h).

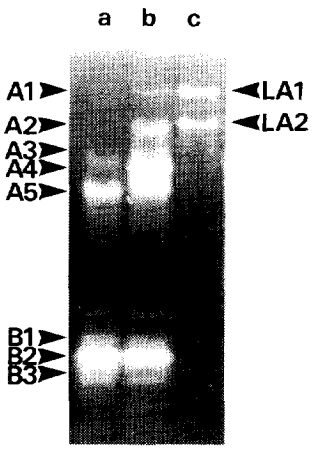

Fig. 3. Effect of reducing agents on the poplar wood α-amylase electrophoretic profile. Crude enzyme from wood was depleted of 2-mercaptoethanol by desalting (lane a) and subsequently made 10 mM in DTT (lane b) before the samples were applied to gels containing amylopectin. Lane c, DTT-reactivated, purified α-amylase from leaves. The labelling of the bands is the same as in Fig. 2. In addition, the position of three bands with β-amylase activity is indicated (B1–B3).

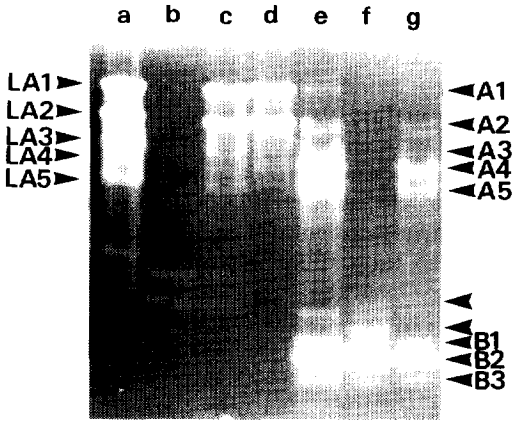

Fig. 4. Poplar endoamylase electrophoretic banding pattern as affected by Mg^{2+}. Samples of purified α-amylase from poplar leaves (lanes a–d) or complete protein extracts from wood (lanes e–g) were passed through desalting columns in a buffer solution comprising 0.1 mM EDTA instead of $MgCl_2$ (lanes b and f). These samples were transferred into the original $MgCl_2$-buffer by a second passage through the desalting column (lanes c and g). DTT to a concentration of 10 mM was added to the sample of lane c (lane d). The samples were stored at 4° for 16 hr before they were electrophoresed on gels containing amylopectin. The labelling of the bands is the same as in Fig. 2 and Fig. 3. The position of the EDTA-induced bands of the wood amylase is marked by arrows.

Substrate specificity

Some common soluble polysaccharides were degraded by the purified amylase from poplar leaves to almost the same extent (Table 2). The complete lack of activity with pullulan, maltose, maltotriose, and maltotetraose shows that the enzyme was free of debranching enzyme, α-glucosidase, and D-enzyme. A very low activity of less than 1% compared with soluble starch was observed with the specific substrate of β-amylase, the PNP-maltooligosaccharides of the Testomar test kit.

Table 2. Substrate specificity of the purified α-amylase from poplar leaves

Substrate	Relative activity
Soluble starch	100
Amylopectin	96.4 ± 10.1*
Amylose	87.3 ± 8.9
β-Limit dextrin	90.0 ± 16.5
Pullulan	n.d†
Testomar	0.5 ± 0.2
Maltose	n.d.
Maltotriose	n.d.
Maltotetraose	n.d.
Starch grains (potato)	4.3 ± 0.8
Starch grains (corn)	17.5 ± 3.6

The activity tests with starch grains as substrate were run at pH 8 whereas all other assays were carried out at pH 6.5. The relative activity refers to the activity (as %) with soluble starch from potato as substrate.

* Values are means ± s.d., n = 3.

† n.d. = no activity detectable.

Starch granules were also readily attacked by purified poplar amylase (Table 2). Granules from corn were degraded at *ca* fourfold higher rate than granules from potato. An explanation for this effect cannot be given because starch granules from commercial sources were used, but it may be speculated that the difference is due to the large size and the resulting relatively small surface area of the potato granules [19]. The activity data of Table 2 with soluble polysaccharides as substrates are not directly comparable with the degradation rates of starch granules because the glucans released from the latter substrate were digested with amyloglucosidase before glucose was quantified, while the degradation of the other substrates was directly determined as reducing power with the dinitrosalicylic acid reagent.

Taken together, the effects of various substrate types on the activity of the purified leaf amylase is consistent with the classification as α-amylase. This conclusion is supported by the fact that the partly purified endoamylase from poplar wood showed a very similar substrate specificity. In this case, the identification as α-amylase was verified by HPLC product analysis [11].

pH Optimum

The effect of pH on the amylase activity was influenced by the kind of substrate and the assay conditions (Fig. 5). With starch azure, the highest reaction rate was observed at pH 6.5. The removal of 2-mercaptoethanol reduced the activity but did not affect the pH response. In contrast, the optimum was shifted to pH 7.3 in the presence of

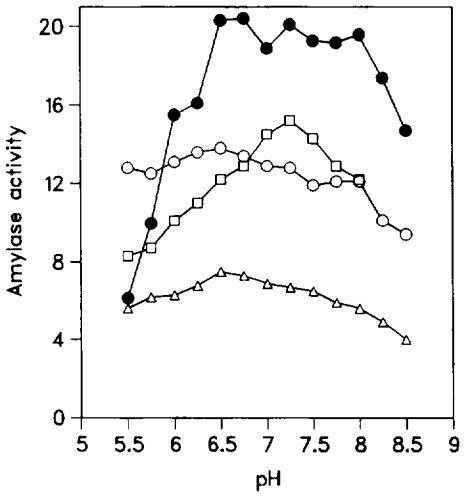

Fig. 5. The pH-effect on the activity of purified poplar leaf α-amylase. The activity was tested at 4° using corn starch granules as substrate (●) or at 30° with starch azure (open symbols). The enzyme was directly applied to the test mixture (○) or the reductant (2-mercaptoethanol) was previously removed by a passage through a desalting column (△). In another test series, DTT in a concentration of 10 mM was present in the incubation mixture (□). The activity is given as units × 10^3 (starch azure) or nmol hr^{-1} (corn starch).

a high concentration of DTT. The degradation of starch grains by α-amylase showed a broad pH response with a plateau of maximal activity between pH 6.5 and 8.0. The assays with granules were run at 4° to prevent oxidative inactivation during the extended incubation period of 20 hr (see below). The reasons for the relatively low sensitivity to pH and the dependence of the pH-response on the kind of substrate are not yet clear, but it is tempting to speculate that the individual electrophoretic forms of the enzyme may show different pH optima and substrate specificities.

Effects of reducing agents

Purified α-amylase was remarkably stable at 4° in the presence of 10 mM $MgCl_2$ and 10 mM 2-mercaptoethanol. Only minor losses of activity were observed within three weeks of storage under these conditions. The removal of the reductant by a passage through a desalting column led to the loss of 80–90% of the activity within 16 hr at 4°. The inactivation was partly reversed by incubation with SH-active compounds (Table 3). The dithiols DTT and DTE were most effective in reconstituting the activity. A further activation was achieved by thioredoxin (TR) in combination with DTT. The effect of DTT was saturated at 10 mM and concentrations of more than 40 mM were inhibitory (results not shown).

The effects of DTT and TR, especially the temperature-dependence of the reactivation, were analysed in more detail (Fig. 6). At 4°, the activity changes were slow: the enzyme was not fully reactivated within 7 hr (Fig. 6A). After further incubation of the samples for 13 hr, the same activity as in untreated control samples was detected. The complete reactivation with DTT alone was not achieved under these conditions (results not shown). Poplar α-amylase activity was not stable at 21° in the presence of 2-mercaptoethanol; a loss of activity of *ca* 70% was observed within 7 hr (Fig. 6B). The enzyme was fully inactivated with 5 hr in the absence of any reductant at this temperature whereas the addition of DTT and TR led to a nearly complete reactivation during this period. At 30°, the presence of DTT alone was not sufficient to stabilize the activity (Fig. 6C). The enzyme was nearly completely reactivated and stabilized over several hours at this temperature only in the presence of DTT and TR.

Table 3. Reactivation of oxidized α-amylase from poplar leaf by reducing agents

Reductant	Activation (n-fold)
–	1
TR†	2.0 ± 0.4*
GSH	3.1 ± 1.4
DTE	6.6 ± 1.4
DTT	7.2 ± 1.5
DTT + TR	12.9 ± 1.8

Samples of α-amylase were depleted of 2-mercaptoethanol by desalting and then kept without reductant for 16 hr at 4°. The activity was restored by addition of the reductants to a concentration of 5 mM (50 μg ml^{-1} TR). The activity was measured with starch azure as substrate.

* Mean ± s.d., n = 3.

† TR = thioredoxin, GSH = glutathione, DTE = dithioerythritol, DTT = dithiothreitol.

The removal of the reductant and the treatment with DTT and TR also affected the banding pattern in PAGE-zymograms. Separations with enzyme samples of the experiment of Fig. 6A after a further storage at 4° for 16 hr are shown in Fig. 2, lanes a–d. The main bands, LA1–LA5, are only detectable with the mercaptoethanol- and Mg^{2+}-stabilized enzyme (Fig. 2, lane a). Only traces of two bands, LA3 and LA4, are found in the absence of reducing agents (Fig. 2, lane b). In DTT- and TR-treated samples, the upper bands (LA1–LA2) were more pronounced whereas the bands LA4 and LA5 were absent (Fig. 2, lanes c and d). Other enzyme samples were incubated for 1 hr at 30° in the presence or absence of reductants; they were then immediately applied to electrophoresis gels (Fig. 2, lanes e–h). The staining intensity of the amylase bands in control samples, which were desalted and incubated in buffer C, was clearly reduced, and other bands with high electrophoretic mobility appeared (lane e). The same bands were also induced by chelation of divalent cations (see below). Hardly any bands are detectable after 1 hr at 30° in the absence of reductants (Fig. 2, lane f). Again, the treatment with DTT and TR resulted in the activation of the upper bands (Fig. 2, lanes g and h).

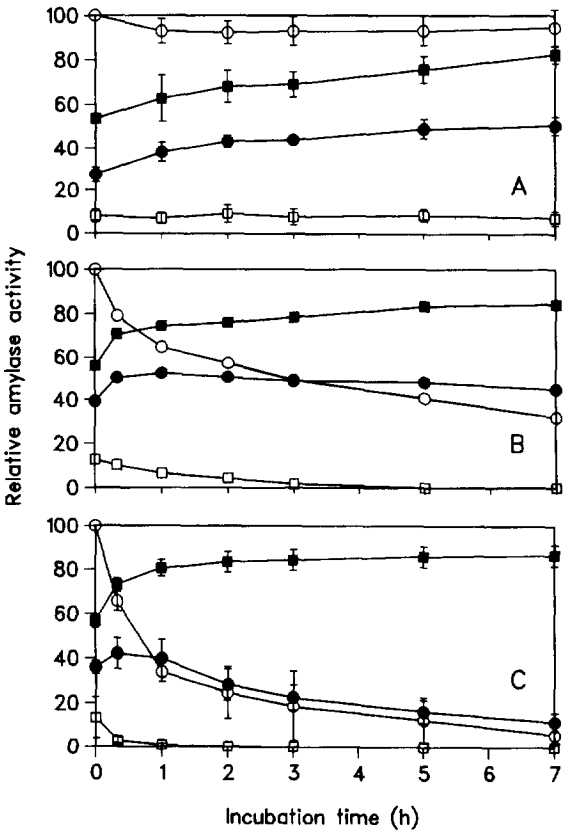

Fig. 6. Activation of poplar leaf α-amylase by DTT and TR. Samples of the purified enzyme were depleted of 2-mercaptoethanol by desalting, stored for 16 hr at 4°, and then reactivated by incubation with 10 mM DTT (●), 10 mM DTT and 100 μg ml^{-1} TR (■), or without reducing agent (□). Another enzyme sample was passed in parallel through the same desalting column but in the storing buffer C comprising 2-mercaptoethanol (○). The incubations were carried out at 4° (panel A), 21° (B), or 30° (C). Samples were removed at the indicated times and tested for amylase activity with starch azure as substrate. The data are given as % of the activity of the enzyme in storing buffer at the beginning of the incubations. The bars indicate s.d. of three experiments. The values of panel B are the mean of two measurements.

The same effects of DTT were observed with the main endoamylase from poplar wood (Fig. 3). Desalting in a medium without 2-mercaptoethanol resulted in the inactivation of the upper amylase bands which were previously designated A1 and A2 [11]. These bands could be restored by DTT (Fig. 3, lane b). For comparison, the DTT-reactivated α-amylase from leaves is shown in Fig. 3, on lane c.

Effects of divalent cations

Another factor that affects α-amylase activity is the binding of divalent cations, especially of Ca^{2+} [2]. Purified α-amylase from poplar shows remarkable stability in the presence of Mg^{2+}. Desalting by a passage through a PD-10 gel filtration column in a Mg^{2+}-free medium led to rapid inactivation. This effect was partly prevented by the subsequent transfer of the enzyme into a medium comprising $MgCl_2$. This treatment resulted in major changes of the electrophoretic profile (Fig. 4). Only two or three band of high electrophoretic mobility but not bands LA1–LA5 were detected in the Mg^{2+}-free sample (Fig. 4, lane b). After transfer into the Mg^{2+}-containing solution, the upper bands, especially LA1 and LA2, are still present (Fig. 4, lane c). Addition of DTT eliminated the fast migrating activity bands (Fig. 4, lane d). The same effects were observed with the α-amylases in crude enzyme extracts from poplar wood (Fig. 4, lanes e–g). The β-amylase bands in the wood extract were not affected by the same procedure.

Table 4. Reactivation of oxidized and Mg^{2+}-depleted α-amylase

Treatment for reactivation	Relative activity, with desalting solution	
	EDTA + meSH	EDTA − meSH
–	17 ± 8*	5 ± 2
$MgCl_2$	40 ± 10	14 ± 9
$CaCl_2$	80 ± 4	33 ± 21
$MgCl_2$ + DTT + TR†	120 ± 31	97 ± 18
$CaCl_2$ + DTT + TR	142 ± 15	128 ± 10
DTT + TR	84 ± 30	78 ± 9

Mg^{2+} was chelated in samples of purified poplar leaf α-amylase by addition of EDTA to a concentration of 10 mM and subsequent desalting in buffer solution comprising 0.1 mM EDTA instead of Mg^{2+} (+ meSH). Other samples were desalted in the same medium but without 2-mercaptoethanol (− meSH). After 16 hr at 4°, the divalent cations and DTT were added to a concentration of 10 mM (TR: 50 μg ml^{-1}). The activity was then tested with starch azure. The relative activity refers to the activity (as %) of a third amylase sample which was desalted in parallel in storage buffer comprising Mg^{2+} and 2-mercaptoethanol.

* Mean ± s.d., n = 3.

† DTT = dithiothreitol, meSH = 2-mercaptoethanol, TR = thioredoxin.

The role of divalent cations in combination with reducing agents in reconstituting α-amylase activity was further analysed in more detail with the purified enzyme from leaves (Table 4). For this purpose, enzyme samples were treated with EDTA and passed through desalting columns to remove $MgCl_2$ and/or mercaptoethanol and the effects of reducing agents as well as Ca^{2+} and Mg^{2+} were subsequently determined. The activity was compared with samples which were passed through the same columns but in the original storage solution (buffer C) comprising Mg^{2+} and 2-mercaptoethanol. The removal of both Mg^{2+} and 2-mercaptoethanol led to an almost complete inactivation. The loss of activity was only partially reversed by divalent cations alone, but most of the activity was reconstituted by DTT and TR. The addition of Ca^{2+} in combination with DTT and TR resulted in an

about 1.3-fold activation over enzyme samples in buffer C. These effects were less pronounced when only Mg^{2+} but not the reductant was removed from the enzyme samples (Table 4, left column). Obviously, a significant part of the activity was stabilized by 2-mercaptoethanol even in the absence of divalent cations.

DISCUSSION

The poplar leaf α-amylase activity was purified 17 700-fold over the crude extract to a specific activity of about 170 μmol min^{-1} mg^{-1} (Table 1). This specific activity is considerably higher compared to preparations from leaves of sugar beet [20], spinach [6], and *Arabidopsis* [21] but less than the specific activities of purified endoamylases from pea shoots and cotyledons [5, 16] and pea leaves [15]. The reasons for the differences are not known, but the activation state of the purified enzymes has to be considered in this respect (see below).

The poplar leaf enzyme can be clearly identified as α-amylase by its substrate specificity. In addition, it shares many properties with the α-amylase from poplar wood: the banding pattern in native PAGE is nearly the same (Fig. 3); both enzymes are not bound to anion exchanger at pH 8.0 and both are retarded on starch grain affinity columns (Witt, W., unpublished results); both enzymes attack starch granules [11], and they respond in a similar way to divalent cations and reducing agents (Fig. 3 and Fig. 4). The complete purification of the wood amylase and the analysis of structural parameters will be necessary to decide on the identity of both enzymes.

Purified poplar leaf endoamylase migrates as a single band (M_r 44 000) on SDS-PAGE gels. Bands in the same range (41–45 k) were also observed with purified α-amylases from other plant sources [5, 15, 16, 20, 22]. Additional α-amylase isoforms of higher apparent M_r were recently identified in *Araucaria* [10] and rice [23]. It is generally assumed that α-amylases are monomeric proteins [5, 15].

In spite of its relatively simple structure, poplar α-amylase shows a complex pattern of multiple forms in native PAGE. Similar effects were observed with pea cotyledon α-amylase [5] and endoamylase from mature pea leaves [15]. This electrophoretic polymorphism is probably not due to post-translational modifications as in some cereal amylases [8, 9, 24] and *Araucaria* [10] because the banding pattern was reversibly shifted by treatments with EDTA, divalent cations, and reductants (Fig. 2 and Fig. 4). Beers and Duke [5] suggested that divalent cations are lost from α-amylase during electrophoresis, allowing some amylase to migrate farther than others. Changes of the net charge alone are probably not responsible for the observed effects, because reducing agents also affected the banding pattern of poplar leaf amylase (Fig. 2 and Fig. 4). The presence of multiple amylase bands is therefore more consistent with the assumption that conformational modifications of the protein structure were induced by the reversible loss of divalent cations or by the pretreatment with reductants. The multiple conformers may have different affinity to the immobilized amylopectin in the gel matrix and they thus are differently retarded throughout the electrophoresis.

The presence of reducing agents was an essential factor to stabilize the poplar leaf endomylase. Storage under air without reductant led to the inactivation of the purified enzyme within several hr. Thioredoxin in combination with DTT was the most effective reducing agent to reactivate the oxidized enzyme (Table 3). Similar effects were observed with debranching enzyme from rice seeds [25], spinach leaves [26], sugar beet leaves [20], and to a minor extent with chloroplastic amylase from spinach [27]. In contrast, α-amylase is usually considered as not dependent on SH-stabilizing agents, and the treatment with chemical reductants and TR had no effects on the purified endoamylases from several plant sources [6, 15, 20] and on the starch degradation in isolated chloroplasts [28]. This apparent contradiction to the results presented here may be explained by the assumption that the purified amylases from other plant sources were in the fully activated state. The purification of α-amylase is usually carried out in media comprising Ca^{2+} and a potent reductant. Accordingly, the treatment with SH-oxidizing agents resulted in the reversible inactivation of the purified pea leaf α-amylase [15].

The α-amylase in poplar wood exhibits very similar responses to reducing agents and divalent cations as the leaf enzyme (Figs 3 and 4). Reduced glutathione was detected in high concentration in the xylem of cold-acclimatized poplar twigs [29], and the activity of glutathione reductase started to increase in autumn [30] concomitant to the onset of the starch degradation in this tissue. The appearance of two forms of α-amylase (A1 and A2) was consistently associated with phases of rapid conversion of starch into soluble carbohydrates [13]. The same amylase forms exhibited high affinity to immobilized amylopectin and were preferentially reactivated by reducing agents (Figs 3 and 4). It is therefore proposed that reductants may be also involved in the *in vivo* activation of these amylolytic enzymes.

EXPERIMENTAL

Plant material. Fully expanded leaves from 14-year-old poplar trees (*Populus* × *canadensis* Moench '*robusta*') were harvested in September 1993; in June to August 1994, and in June 1995. The material was free of any macroscopically detectable microbial infections. After removing the midrib, the leaves were frozen in liquid N_2 and stored at − 75°. The trees are growing in the Botanical Garden of Kiel University. Wood samples from four-years-old twigs of the same trees were harvested and extracted for amylase activity as previously reported [13], except that 0.1 M Hepes/KOH, pH 8, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol was used as extraction medium.

Purification of α-amylase activity from leaves. Frozen leaves (250 g) were ground with mortar and pestle in liquid N_2. The material was transferred into 1.5 l of buffer

A (0.1 M Tris/HCl, pH 8, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol) and 62.5 g polyvinylpolypyrrolidone were added. The leaf tissue was further disrupted by three full-speed treatments (30 sec) with an Ultra-Turrax homogenizer. The suspension was chilled on ice for 20 min with occasional shaking before the cell debris was removed by filtration through 2 layers of Miracloth and centrifugation at 12000 *g* for 30 min. The supernatant was mixed with 80 g of preswollen DEAE-cellulose (Servacel DEAE 23, Serva) in buffer A and agitated for 15 min. The ion exchanger was then removed by filtration under vacuum. Solid $(NH_4)_2SO_4$ was added to 45%. The ppt. was collected after 1 hr by centrifugation as above, dissolved in buffer A (100 ml), and centrifuged again. The supernatant was passed through a column (15 × 1.6 cm) of Q Sepharose ff (Pharmacia), which had been equilibrated with the same soln. The protein in the effluent was precipitated by addition of solid $(NH_4)_2SO_4$ to 50% satn. After 16 hr, the ppt. was collected by centrifugation at 40000 *g* for 20 min and dissolved in 15 ml buffer B (20 mM Hepes/KOH, pH 8, 25 mM $MgCl_2$, 10 mM 2-mercaptoethanol). The soln was dialysed for 5 hr against 600 ml of the same buffer and was then applied to a column (55 × 2.6 cm) packed with starch granules from potatoes (ICN Biomedicals). The granules had been washed 4 times with H_2O and 2 times with buffer B before they were filled into the column. Elution was carried out at a flow rate of 20 ml hr^{-1} while fractions of 10 ml were collected. Amylase activity and the protein content were measured in each fraction; in some cases, the amylase isoenzyme profile was also analysed. Fractions with amylase activity and a low protein content (16–46 in Fig. 1) were pooled and concentrated by ultrafiltration through YM10-membranes (Amicon). The buffer was changed to buffer C (50 mM Hepes/KOH, pH 8, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol) by dilution and concn on the same filter. The enzyme soln (*ca* 2 ml) was layered on a column (95 × 1.6 cm) of Sephacryl S-300-HR (Pharmacia) in buffer C. The column was eluted at a flow rate of 5 ml hr^{-1} and fractions of 5 ml were collected. Each fraction was analysed by SDS-PAGE and tested for amylase activity. Fractions which showed a single band (M_r 44000) were pooled and passed through a second Q Sepharose-column (8 × 1 cm) in buffer C with a flow rate of 50 ml hr^{-1}. All steps were carried out at 0–4°. Protein was determined by the Coomassie dye-binding assay [31] with bovine serum albumin as standard. The purified enzyme was stored at 4° and subjected within 10 days to the different kinetic experiments. In some cases, a partly purified (1050-fold) amylase was used.

Determination of the effects of divalent cations and reductants. $MgCl_2$ and/or 2-mercaptoethanol was removed from the enzyme samples by a passage through desalting columns (PD-10, Pharmacia) according to the instructions of the manufacturer. The buffer soln to equilibrate the columns and to elute the enzyme contained 50 mM Hepes/KOH (pH 8) in all cases. Variations of the procedure are outlined in the legends of figures and tables. Stock solns (0.1 M in Hepes buffer) of chemical reductants for the reactivation of oxidized amylase were set up daily. Recombinant TR (*Escherischia coli*) was obtained from Calbiochem or from Promega-Serva and stored in Hepes buffer (1 mg ml^{-1}) at 4°. If TR and DTT were used in combination, both compounds were preincubated together at 20–22° for 10 min before the amylase solution was added.

Enzyme assays. The tests on amylase activity with soluble polysaccharides as substrates and the assays on maltase and D-enzyme were carried out as previously reported [13, 14] except that Mes/KOH, pH 6.5, was usually used as buffer substance. The activity of β-amylase was tested with PNP-maltooligosaccharides as substrates (Testomar test kit, Behring Werke) by the procedure of ref. [32] with slight modifications [14]. The release of the Remazol Brilliant Blue-dye from starch azure (Sigma) for determination of endoamylase activity was measured at a substrate concn of 0.9% in 1 ml reaction mixtures as previously reported [11] except that the reaction was terminated by addition of 0.25 ml 50% TCA and that Hepes/KOH (pH 8) at 50 mM was usually used as buffer substance. Some modifications of the assay conditions are given in the legends of figures and tables. The tests were run in duplicate and at 30° if not stated otherwise.

The degradation of starch granules by poplar amylases was quantified as described elsewhere [12]. Briefly, the incubation was stopped by centrifugation to remove the granules. The released glucans in the supernatant were converted to Glc by digestion with amyloglucosidase. Glucose was measured as previously reported [13]. Starch granules from potatoes (ICN Biomedicals) as well as from corn (Merck) were used as substrates.

Electrophoresis. Proteins were separated under reducing, denaturing conditions by SDS-PAGE using 12%-slab gels under a Laemmli-buffer system [33]. Native PAGE using 6.5% gels containing 0.05% (w/v) amylopectin to separate different endoamylase forms and the visualization of bands by negative staining with I_2 was carried out as described elsewhere [13].

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REFERENCES

1. Steup, M. (1988) in *The Biochemistry of Plants* (Preiss, J. ed.) Vol. **14**, p. 255. Academic Press, San Diego.
2. Beck, E. and Ziegler, P. (1989) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 95.
3. MacGregor, E. A. (1993) *Starch* **45**, 232.
4. Svensson, B. (1994) *Plant Mol. Biol.* **25**, 141.
5. Beers, P. E. and Duke, S. H. (1990) *Plant Physiol.* **92**, 1154.
6. Okita, T. W. and Preiss, J. (1980) *Plant Physiol.* **66**, 870.
7. Jacobsen, J. V., Hanson, A. D. and Chandler, P. C. (1986) *Plant Physiol.* **80**, 350.

8. Sticher, L. and Jones, R. (1991) *Plant Physiol.* **97**, 936.
9. Søgaard, M., Olsen, F. L. and Svensson, B. (1991) *Proc. Natl Acad. Sci. USA* **88**, 8140.
10. Acevedo, E. and Cardemil, L. (1994) *Physiol. Plant.* **92**, 149.
11. Witt, W. and Sauter, J. J. (1995) *J. Plant Physiol.* **146**, 15.
12. Witt, W. and Sauter, J. J. (1995) *J. Plant Physiol.* **146**, 35.
13. Witt, W. and Sauter, J. J. (1994) *J. Plant Physiol.* **143**, 625.
14. Witt, W. and Sauter, J. J. (1994) *Physiol. Plant.* **92**, 9.
15. Ziegler, P. (1988) *Plant Physiol.* **86**, 659.
16. Hirasawa, E. and Yamamoto, S. (1991) *Planta* **184**, 438.
17. Gerbrandy, S. J. (1974) *Biochim. Biophys. Acta* **370**, 410.
18. Subbaramaiah, K. and Sharma, R. (1985) *J. Biochem. Biophys. Methods* **10**, 315.
19. Walker, G. W. and Hope, P. M. (1963) *Biochem. J.* **86** 452.
20. Li, B., Servaites, J. C. and Geiger, D. R. (1992) *Plant Physiol.* **98**, 1277.
21. Lin, T.-P., Spilatro, S. R. and Preiss, J. (1988) *Plant Physiol.* **86**, 251.
22. Koizuka, N., Tanaka, Y. and Morohashi, Y. (1990) *Plant Physiol.* **94**, 1488.
23. Chen, M.-H., Liu, L.-F., Chen, Y.-R., Wu, H.-K. and Yu, S.-M. (1994) *Plant J.* **6**, 625.
24. Sopanen, T. and Laurière, C. (1989) *Plant Physiol.* **89**, 244.
25. Yamada, J. (1981) *Carbohydr. Res.* **90**, 153.
26. Ludwig, L., Ziegler, P. and Beck, E. (1984) *Plant Physiol.* **74**, 856.
27. Pongratz, P. and Beck, E. (1978) *Plant Physiol.* **62**, 687.
28. Stitt, M. and Heldt, H. W. (1981) *Biochim. Biophys. Acta* **638**, 1.
29. Sagisaka, S. (1986) *Plant Cell Physiol.* **27**, 1209.
30. Sagisaka, S. (1985) *Plant Cell Physiol.* **26**, 1135.
31. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248.
32. Ziegler, P. (1990) *J. Plant Physiol.* **136**, 580.
33. Laemmli, U. K. (1970) *Nature* **227**, 680.