**Homology among Multiple Extracellular Peroxidases from *Phanerochaete chrysosporium***

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The extracellular peroxidases of *Phanerochaete chrysosporium* were separated into 21 proteins by analytical isoelectric focusing. Fifteen of these enzymes oxidized veratryl alcohol (lignin peroxidases) in the presence of H$_2$O$_2$. Six enzymes were Mn(II)-dependent peroxidases. The Mn(II)-dependent enzymes appeared and reached their maximal activity earlier than the lignin peroxidases in the cultures. Peptide mapping, amino acid analysis, and reaction against specific antibodies showed that all the Mn(II)-dependent peroxidases were probably products of one gene. A great degree of homology was also present among the various lignin peroxidases.

Biological lignin degradation has gained much attention since the original discovery of an enzyme from culture filtrates of *Phanerochaete chrysosporium* able to partially depolymerize lignin and oxidize several lignin-related aromatic compounds (1-3). During the last 3 years, this enzyme has been called a lignin degrading enzyme (1), lignin degrading H$_2$O$_2$-dependent peroxidase (3), diaryl propane oxidase (2), and ligninase (4). Recently this enzyme was shown to be a peroxidase (5, 6) which oxidizes different aromatic substrates by a one-electron transfer mechanism. The existence of multiple molecular forms of this lignin peroxidase has been reported (7-9). The lignin peroxidase can oxidize molecules that are not normally its substrates through electron transfer mediators like veratryl alcohol and dimethoxybenzene (10-12).

In culture filtrates of *P. chrysosporium* Kuwahara et al. (13) also found another type of peroxidase that is Mn(II)-dependent. This enzyme has been purified and characterized (14, 15). It oxidizes Mn(II) to Mn(III), which is able to oxidize various substrates. This enzyme has also been separated into multiple molecular forms (7, 15).

The question of homology among the multiple forms of *P. chrysosporium* peroxidases and the function of the hemoproteins separated thus far (7, 8, 15) has not been satisfactorily answered. In this paper we show that, in the culture fluid of *P. chrysosporium*, more than 20 extracellular hemoproteins can be found which all have peroxidative activity. Furthermore, on the basis of reaction specificity, immunological properties, peptide mapping, and amino acid composition, these peroxidases can be divided into two basic groups.

**MATERIALS AND METHODS**

Production of Peroxidases—*P. chrysosporium* (ATCC 24725) was grown in nitrogen- or carbon-limited cultures. Medium composition was according to Kirk et al. (16), with 7-fold trace element concentration (8). The cultures were inoculated with a spore suspension. C-limited cultures were grown on a rotary shaker (150 rpm, 2.5 cm) in 1000-ml Erlenmeyer flasks (600 ml of medium) for 48 h in air at 37 °C, after which the peroxidases were induced as described previously (17). N-limited cultures were grown in non-agitated cultures (20 ml of medium in 290-ml flasks) since under agitated conditions relatively more polysaccharide material, which interfered with enzyme purification, was produced. Veratryl alcohol (1.5 mM) was added at the time of inoculation. The flasks were flushed with pure oxygen and incubated at 37 °C up to 120 h.

Partial Purification of Peroxidases—The extracellular enzyme solution was recovered by filtration through a glass-fiber filter and concentrated by ultrafiltration through an Amicon PM-10 membrane. The enzyme concentrations were dialyzed against 5 mM potassium phosphate, pH 7.25, bound to DEAE Bio-Gel A anion exchanger in a 1.5 × 5-cm column and eluted with 0.5 M NaCl in 5 mM phosphate. The resulting enzyme solution was again concentrated by ultrafiltration and the NaCl removed by gel filtration through a 1.5 × 60-cm Sephadex G-75 column using 20 mM acetic acid as eluent. The peroxidases were concentrated to 2 mg/ml protein and stored at 4 °C until further purification.

*Isoelectric Focusing (IEF)*—The partially purified peroxidases were separated by analytical isoelectric focusing on a 0.5% agarose gel. Bio-Rad amphylates 3/5 and 4/6 were used in a 1:1 ratio. After focusing 2–5 h at constant voltage of 600 V, the peroxidases were identified either by direct visualization of the yellow color (heme) or by activity staining using 4-chloro-1-naphthol (Bio-Rad Immun-Blot) as substrate. The activity staining was carried out in 50 mM sodium tartrate at pH 3.0 or in 50 mM sodium succinate buffer in the presence of Mn(II) at pH 4.5. The staining was carried out in the presence of 50 μM hydrogen peroxide. For the measurement of specific activities and reaction specificities, the major hemoproteins as visualized by yellow color were cut out of the gel, 100 μl of water were added to each gel fragment, and the mixture was dialyzed against 20 mM 2,2-dimethyl succinate, pH 4.5, to remove the amphylates.

**Peptide Mapping, Cleavage at Methionine**—Peptide mapping was carried out by cyanogen bromide cleavage of proteins in polyacrylamide gels followed by second-dimension SDS-electrophoresis (18). 1-2 μg of C- or N-limited partially purified peroxidases were separated by isoelectric focusing in polyacrylamide gel (T=5, C=3) in gel rods (4 × 140 mm) at 500 V for 8,000-10,000 V-h. LKB ampholines, pH 2.5-6 (150 μl) and 4-6 (150 μl) were used for 10 ml of the acrylamide solution. Isoelectric focusing under denaturing conditions (9 M urea, 1% Nonidet P-40) was performed as described (19) with LKB ampholines, pH 3-10, 2.5-4, and 4-6 in the ratio 1:2:1 in gel rods (4 × 140 mm) at 500 V for 10,000 V-h. A rather high concentration of cyanogen bromide (70-100 mg/ml) was found to be necessary for cleavage of proteins in nondenaturing IEF gels. The peptides formed were separated by second-dimension SDS electrophoresis in polyacrylamide gels (T=15, C=1) and detected by silver staining (20).

**Cleavage at Tryptophane**—The peptide mapping method (21) was adapted for small amounts of protein. The peroxidases were separated by analytical isoelectric focusing and cut from the gel. The enzymes were eluted from the gel with water and dialyzed against 20 mM acetic acid. The peptide mapping was carried out with the purified enzymes using 2-(2-nitrophenylsulfenyl)-3-methyl-3-bromindole (BNPS-SKFS).

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skatole) which selectively cleaves tryptophanyl bonds. 20 mg of BNPS-skatole (recrystallized from petroleum ether and stored in 20-mg quantities at -20 °C) was dissolved in 2 ml of acetic acid containing 1 mg/ml phenol. 70 µl of this solution were mixed with 30 µl of the protein sample in acetic acid. The mixture was left for 48 h at room temperature in the dark. Then 20 µl of 5 M Tris were added followed immediately by 800 µl of ethyl acetate and mixed. The phases were separated by short centrifugation, and the upper layer was discarded. The lower phase was once more extracted with 800 µl of ethyl acetate. 10 µl of 20% SDS were then added and mixed with the water phase. Traces of ethyl acetate were evaporated, and the sample (volume now approximately 20 µl) was dialyzed and prepared for electrophoresis as usual. A gradient gel (10–30%) with the Laemmli system was used. Molecular weight standards were obtained from Pharmacia. Detection was by silver staining (20).

Preparation of Antibodies—20 mg of a crude peroxidase preparation from C-limited cultures were separated by preparative isoelectric focusing. The band corresponding to the lignin peroxidase with an isoelectric point of 4.65 was removed from the gel, eluted with water, and dialysed against 5 mM potassium phosphate, pH 7.25. The protein solution was mixed with an equal volume of Freund’s complete adjuvant (Difco), sonicated, and injected into rabbits. Booster injections of protein mixed with incomplete adjuvant were given every 2 weeks. After 8 weeks, a positive reaction was observed in Ouchterlony double-diffusion assays, and 25 ml of blood were removed from the animals. The IgG fraction was purified by chromatography on Affigel Blue (Bio-Rad) and subsequent ammonium sulfate precipitation (35%). The pellet was redissolved in phosphate-buffered saline (50 mM phosphate, 500 mM NaCl, pH 7.4) and dialyzed extensively against the same buffer. Antibodies were purified by adsorption on electrophoretically homogenous peroxidase immobilized via teryl chloride (22) on glycerol-CPG (1000 A, Fluka) and eluted with 100 mM glycine HC1, pH 2.3. After adjusting the pH to 7.4 these antibodies were used in immunoblotting.

Immunoblotting—Enzymes from carbon- and nitrogen-limited cultures were separated by analytical isoelectric focusing as described above. Proteins were transferred to nitrocellulose by capillary blotting. Staining of the agarose gel after blotting with Coomassie Blue did not reveal any protein bands indicating quantitative transfer. Blocking, incubation with first and second antibody, and washings were performed as described for an enzyme-linked detection system (Bio-Rad Immun-Blot”) with the exception that iodinated second (anti-rabbit) antibodies were used. Filters were exposed at -70 °C on x-ray films (Fuji) for 2–5 days.

Analytical Methods—Lignin peroxidase activity was measured according to Tien and Kirk (3). One unit of enzyme oxidized 1 µmol of veratryl alcohol to veratrdehyde in min at room temperature. Mn(II)-dependent peroxidase was measured according to Kuwahara et al. (13) using phenol red as a substrate. The reaction time was 5 min and 0.5–2.0 µg of protein were used for the test. For oxidation of phenol red, the oxidations were carried out at pH 3.0 in 20 mM sodium tartrate in the presence and absence of 0.4 mM veratryl alcohol. The reaction conditions were as in measurement of the Mn(II)-dependent peroxidase.

Soluble protein was measured by the method of Bradford (23) using bovine serum albumin as standard. For amino acid analysis, peroxidases from C- or N-limited cultures were purified by chromatofocusing (7) or by preparative agarose-Sephadex isoelectric focusing (24). The enzymes were detected by either their absorbance at 405 nm (chromatofocusing) or by their yellow color (IEF) and subsequently dialyzed against 20 mM acetic acid (pH 3) and lyophilized. The protein was redissolved in constant boiling HCI and hydrolyzed in sealed tubes for 24 h at 110 °C under nitrogen. Appropriate aliquots were applied to an amino acid analyzer (Biotronic LC 6000E).

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RESULTS

Carbon- and Nitrogen-limited Cultures—The C- and N-limited cultures of P. chrysosporium were harvested when they had reached their maximal lignin peroxidase activity based on veratryl alcohol oxidation. After concentration and preliminary purification (see “Materials and Methods”) the extracellular protein concentrates were separated using analytical agarose isoelectric focusing. Seven major hemoproteins from C-limited cultures and 10 from N-limited cultures could be directly visualized (Fig. 1, lanes B and C). Activity staining at pH 3.0 showed that all the hemoproteins exhibited peroxidase activity (Fig. 1, lanes A and D). From both cultures a total of 16 peroxidases with different isoelectric points were resolved and isolated from isoelectric focusing gels. Band two from N-limited cultures could sometimes be separated into
two. However, this did not occur systematically and since both bands showed similar activities and characteristics they are considered here as one protein.

Reaction Specificity—The major hemoproteins excised from isoelectric focusing gels (Fig. 1) were tested for their activities with veratryl alcohol and phenol red. The specific activities against these substrates are given in Table I. All the hemoproteins of C-limited cultures oxidized veratryl alcohol with minor variations in specific activities. All these enzymes also oxidized phenol red. Oxidation was faster at pH 3.0 than at pH 4.5, and Mn(II) did not affect the rate. For proteins 6 and 7, the phenol oxidation rates were 2 times higher in the presence of veratryl alcohol.

In N-limited cultures, six out of the 10 hemoproteins oxidized veratryl alcohol. Four of the hemoproteins oxidized neither veratryl alcohol nor phenol red. However, in the presence of Mn(II), the oxidation of phenol red by these four enzymes proceeded rapidly. These enzymes are here called Mn(II)-dependent peroxidases, although they oxidized 4-chloro-1-naphthol even without added Mn(II). Phenol red was also oxidized by the six lignin peroxidases but at a considerably lower rate, and Mn(II) had no effect on the oxidation. In the presence of veratryl alcohol, the oxidation of phenol red doubled.

Time Course—We investigated further the time course of the appearance of the different peroxidase species in N-limited cultures. The Mn(II)-dependent peroxidase activity appeared first after 60 h and reached maximal activity 8-10 h later (Fig. 2A). Lignin peroxidase activity appeared 4 h after the appearance of the Mn(II)-dependent peroxidase activity. To correlate the activity with different isoenzymes, the partially purified and concentrated peroxidases were separated by isoelectric focusing at different culture times (Fig. 2B). After 60 h the major protein was the Mn(II)-dependent peroxidase with an isoelectric point of 4.55. After 74 h all the major Mn(II)-dependent peroxidases had reached their maximal concentration, whereas afterwards the lignin peroxidases became the dominant species.

Peroxidase species in addition to those given in Table I were detected when the cultures were harvested after different growth periods. In the early stages (prior to 74 h) Mn(II)-dependent peroxidases with isoelectric points of 4.85 and 4.8 and later (74-100 h) lignin peroxidases with isoelectric points of 3.9, 3.4, and 3.3 were detected in addition to those given in Table I. Altogether, 11 lignin peroxidases and six Mn(II)-dependent peroxidases were present in N-limited cultures. When activity staining was carried out at pH 4.5 in the presence of Mn(II), the C-limited cultures also showed weak Mn(II)-dependent peroxidase activity at pH 4.8-4.9. Moreover, when the C-limited cultures were harvested before the lignin peroxidases had reached their maximal activity, the same Mn(II)-dependent peroxidase species could also be detected as in N-limited cultures (data not shown).

It must be emphasized that the timing of the enzyme recovery has a great influence on the peroxidase pattern obtained. Therefore, variations in the intensity of different peroxidase fractions from one enzyme batch to another usually occurred. Furthermore, after 74 h of cultivation, there was some overlapping between lignin peroxidases and Mn(II)-dependent peroxidases in the pH region of 4.1-4.6. To avoid further complications all the data in this work except those in Figs. 2 and 5 are from the same enzyme batch.

Homology—The large number of different peroxidases in the cultures of P. chrysosporium raises the question of homology between the different protein species. To investigate homology some additional experiments were carried out.

### Table I

| Fraction no.  | pH | Lignin peroxidase | Phenol red oxidation |
|---------------|----|-------------------|----------------------|
|               |    |                   | pH 3.0               | pH 3.0 + VA* + Mn(II) |
|               |    |                   |                      |
| C-limited     |    |                   |                      |
| 1             | 4.55| 0                 | 0                    |
| 2             | 4.55| 0                 | 0                    |
| 3             | 4.55| 0                 | 0                    |
| 4             | 4.55| 0                 | 0                    |
| 5             | 4.55| 0                 | 0                    |
| 6             | 4.55| 0                 | 0                    |
| 7             | 4.55| 0                 | 0                    |

| N-limited     |    |                   |                      |
| 1             | 4.0 | 0                 | 0                    |
| 2             | 4.0 | 0                 | 0                    |
| 3             | 4.0 | 0                 | 0                    |
| 4             | 4.0 | 0                 | 0                    |
| 5             | 4.0 | 0                 | 0                    |
| 6             | 4.0 | 0                 | 0                    |
| 7             | 4.0 | 0                 | 0                    |

*a Numbers refer to Fig. 1.

VA = veratryl alcohol.
The C- and N-limited peroxidases were separated in polyacrylamide gel rods under nondenaturating conditions by IEF. Separation in a second dimension by SDS electrophoresis showed that the N-limited cultures had basically two groups of proteins (Fig. 3A). The more basic Mn(II)-dependent peroxidases with a molecular weight of about 46,000 (13) showed a single band in the second dimension, whereas the more acidic lignin peroxidases were more heterogeneous in their molecular weight. Traces of the more basic lignin peroxidases just below the Mn(II)-dependent peroxidases were also detectable in N-limited cultures (Fig. 3A). A molecular weight of 39,000 has been published for these enzymes (7). The C-limited peroxidases with different isoelectric points consisted of 2–3 protein bands with slightly different molecular weights (Fig. 4A).

After isoelectric focusing the gel rods were incubated with cyanogen bromide, and the resultant peptides were separated according to their molecular weights in a second dimension (Figs. 3B and 4B). All the four major as well as the minor Mn(II)-dependent peroxidases were very similar and showed four fragments having the same molecular weight (bands 1, 2, 4, and 5 in Fig. 3B). More peptide fragments were found among the lignin peroxidases from N-limited cultures, but the individual enzymes were not well separated and therefore the results are difficult to interpret.

Peroxidases 1–4 from C-limited cultures showed identical peptide patterns which were somewhat different from that of peroxidases 5 and 6 (Fig. 4B). During storage the lignin peroxidase with pI 4.65 and 4.6 changed to the enzyme with pI 4.3. In fresh enzyme concentrates the pI 4.3 lignin peroxidase was practically absent.

Because of the poor separation of the acidic lignin peroxidases in the two-dimensional system, these enzymes were separately cleaved by BNPS-skatole. The peptide patterns for the acidic lignin peroxidases were very similar, characterized by two peptides with molecular weights slightly above 20,000 (Fig. 5A). The lignin peroxidase with pI 4.65 gave a pattern very similar to the more acidic enzymes, whereas the pI 4.15 peroxidase differed from the other lignin peroxidases to some extent. The Mn(II)-dependent enzyme showed a different pattern from the lignin peroxidases. The molecular weights in the gradient gel differed from those previously published. C-limited peroxidases showed an apparent molecular weight of 43,000–45,000, like the Mn(II)-dependent enzyme. The acidic lignin peroxidases had a somewhat higher molecular weight.

The amino acid compositions of two lignin peroxidases from C- and N-limited cultures and of two Mn(II)-dependent peroxidases from an N-limited culture are shown in Table II. The data represent only relative values, since the exact molecular weight of the respective protein moiety was unknown due to lack of detailed sugar analysis. However, when a
molecular weight of about 37,000 was assumed, the values were very similar in all the tested peroxidases. As could be expected from their rather acidic isoelectric points, acidic amino acids prevail in all proteins. The major difference in the amino acid composition between lignin peroxidases and the Mn(II)-dependent peroxidases seemed to be in glycine, leucine, and isoleucine.

Further evidence for the presence of two different classes of peroxidases comes from Western blots using polyclonal antibodies against the lignin peroxidase (pI 4.65). In order to exclude nonspecific interactions, the antibodies were purified as described under “Materials and Methods.” As can be seen in Fig. 6, the antibodies reacted exclusively with lignin peroxidase (pI 4.65), which was used as antigen, and with the more basic lignin peroxidases having isoelectric points of 4.3 and 4.5. A much weaker response was observed with the more acidic lignin peroxidases with pI values below 4.3. No reaction occurred with the Mn(II)-dependent peroxidases (Fig. 6, lane D), indicating that these enzymes share no common antigenic sites with the lignin peroxidases. No conclusion can be drawn about the reactivity of the proteins between pI 4.1 and 4.5 from the 100-h N-limited cultures (Fig. 6, lane E), since lignin peroxidases and Mn(II)-dependent peroxidases overlap in older cultures (see Fig. 2B).

**DISCUSSION**

In this study we have separated the crude extracellular peroxidases from *P. chrysosporium* into 21 different hemoprotein species. Six of these enzymes use Mn(II) in oxidation reactions and 15 oxidize veratryl alcohol to veratraldehyde. In previous studies Leisola et al. (7) separated four, Renganathan et al. (9) three, Kirk et al. (8) six, and Paszczynski et al. (15) two enzymes with lignin peroxidase activity. Mn(II)-dependent peroxidase activity in cultures of *P. chrysosporium* was originally discovered by Kuwahara et al. (13). Subsequently Leisola et al. (7) and Paszczynski et al. (15) attributed Mn(II) dependence to at least two enzymes. The difference between these and the present results is probably partly due to incomplete separation and/or inactivation of the minor peroxidase components during purification in previous studies. Furthermore, the timing of peroxidase harvesting also affects the total number of peroxidase species obtained. Isoelectric focusing combined with activity staining proved to be a powerful tool in separation and detection of these enzymes. Comparison of the relative amino acid compositions shows a high degree of homology between the extracellular peroxidases although differences in the content of neutral amino acids exist between the lignin peroxidases and the Mn(II)-dependent enzymes. A somewhat different amino acid composition has recently been published for an Mn(II)-dependent peroxidase from the same organism (15). Peptide mapping of the lignin peroxidases produced under C-limited conditions...
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(424) showed very similar patterns characterized by a group of 6–8 closely spaced large fragments and one or two much smaller peptides. A closely related fragmentation pattern was obtained from two more basic and from the more acidic peroxidases synthesized under N-limited conditions (Figs. 4A and 5A). All these enzymes could also oxidize veratryl alcohol. The Mn(II)-dependent peroxidases were cleaved in a different way (Figs. 4A and 5A). On the basis of these results we suggest that the many extracellular peroxidases of *P. chrysosporium* can simply be divided into two basic groups: Mn(II)-dependent peroxidases and lignin peroxidases.

The difference between the lignin peroxidases and the Mn(II)-dependent enzymes becomes evident from the analysis of Western blots with antibodies raised against the lignin peroxidase with pI of 4.65. Although some lignin peroxidases (pI 4.65, 4.5, and 4.3) are strongly recognized by the antibody, others react more weakly. Since the proteins were transferred from nondenaturing agarose gels, the weaker binding of antibodies is more likely due to differences in structure than denaturation upon transfer to a nitrocellulose membrane. No reaction of antilignin peroxidase antibodies was observed with the Mn(II)-dependent enzymes, demonstrating that these enzymes share no antigenic determinants with lignin peroxidases. This is in accordance with the results of peptide mapping (see above). Lignin peroxidases which reacted weakly with antibodies against lignin peroxidase pI 4.65 may represent either similar (homologous) polypeptides encoded by another gene (isoenzymes) or the same proteins modified post-translationally to a greater extent. Kirk et al. (8) also found a great degree of homology among the six lignin peroxidases after proteolytic cleavage of the individual enzymes.

At present we do not know the reasons for IEF resolution of *P. chrysosporium* peroxidases into so many homologous proteins. Artifacts due to protein-protein interactions during isoelectric focusing can be ruled out because similar results were obtained after isoelectric focusing in 9 M urea, 1% Nonidet P-40 (not shown). Both lignin peroxidase and Mn(II)-dependent peroxidase have been reported to be glycoproteins (3, 15). Charge heterogeneity due to sialic acids of glycoproteins from higher organisms is common. Microorganisms do not possess sialic acids in their glycoproteins, but glycoproteins from higher organisms is common. Microorganisms do not possess sialic acids in their glycoproteins, but charged derivatives of monosaccharides containing phosphate and sulfate groups have been found as constituents of *Dicyostelium* glycoproteins (25, 26). Further work is needed to find out whether varying glycosylation or other post-translational modifications are responsible for heterogeneity of these peroxidases.

It is not clear why *P. chrysosporium* produces two types of extracellular peroxidases. The fact that they appear and reach their maximal activity at different times indicates that they may have different function in lignin degradation. However, so far, the role of Mn(II)-dependent peroxidases in lignin degradation is unclear. The lignin peroxidases have been shown to partially depolymerize lignin (1). Lignin is rapidly degraded to CO₂ in the presence of high concentrations of lignin peroxidases (27). However, these enzymes, which act by one-electron transfer mechanism (5), tend to polymerize lignin in vitro (12). Harvey et al. (28) pointed out that the lignin peroxidases of *P. chrysosporium* have a catalytic cycle similar to horseradish peroxidase. The uniqueness of the lignin peroxidases according to these authors lies in their ability to oxidize both phenolic and nonphenolic substrates. However, these peroxidases alone are unable to degrade lignin completely. The reactions that follow the initial oxidations probably involve reductive as well as aromatic ring cleavage steps which change the equilibrium from spontaneous polymerization to degradation.

REFERENCES

1. Tien, M., and Kirk, T. K. (1983) Science 221, 661–663
2. Gold, M. H., Kuwahara, M., Chiu, A. A., and Glenn, J. K. (1984) *Arch. Biochem. Biophys.* 234, 353–362
3. Tien, M., and Kirk, T. K. (1984) *Proc. Natl. Acad. Sci. U. S. A.* 81, 2280–2284
4. Faison, B. D., and Kirk, T. K. (1985) *Appl. Environ. Microbiol.* 49, 299–304
5. Harvey, P. J., Schoemaker, H. E., Bowen, R. M., and Palmer, J. M. (1985) *FEBS Lett.* 183, 13–16
6. Kula, D., Tien, M., Fee, J. A., and Ondrias, M. R. (1985) *Biochemistry* 24, 3394–3397
7. Leisola, M. S. A., Meissdoerffer, F., Waldner, R., and Fiechter, A. (1985) *J. Biotechnol.* 2, 379–382
8. Kirk, T. K., Croan, S., Tien, M., Mutagh, K. E., and Farrell, R. (1986) *Enzyme Microb. Technol.* 8, 27–32
9. Renganathan, V., Miki, K., and Gold, M. H. (1986) *Arch. Biochem. Biophys.* 241, 304–314
10. Harvey, P. J., Schoemaker, H. E., and Palmer, J. M. (1986) *FEBS Lett.* 195, 242–246
11. Haemmerli, S. D., Leisola, M. S. A., Sanglard, D., and Fiechter, A. (1986) *J. Biol. Chem.* 261, 6900–6903
12. Haemmerli, S. D., Leisola, M. S. A., and Fiechter, A. (1986) *FEBS Microbiol. Lett.* 35, 33–36
13. Kuwahara, M., Glenn, J. K., Morgan, M. A., and Gold, M. H. (1984) *FEBS Lett.* 169, 247–249
14. Glenn, J. K., and Gold, M. H. (1985) *Arch. Biochem. Biophys.* 242, 329–341
15. Paszczynski, A., Huynh, V.-B., and Crawford, R. (1986) *Arch. Biochem. Biophys.* 244, 760–765
16. Kirk, T. K., Schulz, E., Connors, W. J., Lorenz, L. F., and Zeikus, J. G. (1978) *Arch. Microbiol.* 117, 277–285
17. Leisola, M. S. A., Thelan-Wys, U., and Fiechter, A. (1985) *J. Biotechnol.* 3, 97–107
18. Pepinsky, R. B. (1983) *J. Biol. Chem.* 258, 11229–11239
19. Duncan, R., and Hershey, J. W. B. (1984) *Anal. Biochem.* 138, 144–155
20. Morrissey, J. H. (1981) *Anal. Biochem.* 117, 307–310
21. Hunziker, P. E., Hughes, G. J., and Wilson, K. J. (1980) *Biochem. J.* 187, 515–519
22. Nilsson, K., and Mosbach, K. (1980) *Methods Enzymol.* 104, 56–69
23. Bradford, M. M. (1976) *Anal. Biochem.* 72, 248–254
24. Manrique, A., and Laskey, M. (1981) *Electrophoresis* 2, 315–320
25. Ivatt, R. L., Das, O. P., Henderson, E. J., and Robbins, P. W. (1984) *Cell Biol.* 38, 561–567
26. Freeze, H. H. (1985) *Arch. Biochem. Biophys.* 243, 690–693
27. Buswell, J. A., Mollet, B., and Odier, E. (1984) *FEBS Microbiol. Lett.* 25, 295–299
28. Harvey, P. J., Schoemaker, H. E., and Palmer, J. M. (1985) *Annu. Proc. Phytochem. Soc.* 26, 249–250