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# Note

# Mechanism of the adsorption of pancreatic alpha-amylase onto starch crystallites

Valérie M. Leloup [a], Paul Colonna [a] and Guy Marchis-Mouren [b]

[a] *Institut National de la Recherche Agronomique, B.P. 527, F-44026 Nantes 03 (France)*
[b] *Laboratoire de Chimie Biologique Moléculaire de la Nutrition, U.A. 202 C.N.R.S., Faculté des Sciences, Université d'Aix-Marseille, Avenue Normandie Niemen, F-13397 Marseille 3 (France)*

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The mechanism of the adsorption of alpha-amylase onto crystalline starch is still little understood [1–5] despite its involvement in many industrial applications (degradation of starch/saccharides [6], purification of enzymes [3,4]) and digestion phenomena. Nutritionally, the resistance of crystalline starch to alpha-amylase has received attention for both its hypoglycemic [7,8] and fibre-like effects [9–12]. Adsorption onto solid starch has been observed for many alpha-amylases (bacterial [3–5,13], mammalian [1,13]) but examples of non-adsorption have been found (fungal [1,13], bacterial [1,13]) The adsorption mechanism in terms [5] of molecular interactions has not yet been clarified. Studies of the adsorption of alpha-amylase are often complicated by numerous other parameters [1,2]. Thus, the porosity, adsorption surface, crystallinity, and susceptibility to hydrolysis of starch may vary significantly depending on the botanical source or the degree of damage [14]. The aim of this work was to characterise the interactions of alpha-amylase and crystalline starch in a model system in which all of these parameters are controlled.

Starch usually occurs as semi-crystalline structures containing interconnected amorphous and crystalline regions. The crystalline fraction consists of a three-dimensional aggregate of double helices formed from (1 → 4)-α-D-glucan segments. The double helices adopt well-defined arrays (types A or B), the characteristics of which are now well known [15,16]. Ring et al. [17], by spherulitic recrystallisation from concentrated solutions, obtained highly crystalline starch structures, the porosity and surface of which could be determined easily.

Porcine pancreatic alpha-amylase (PPA), which occurs as the isoenzymes PPA-I and PPA-II, is the best known alpha-amylase [18,19] and is responsible for the digestion of starchy materials. PPA-I is composed of three distinct domains

*Correspondence to:* Dr. P. Colonna, Institut National de la Recherche Agronomique, B.P. 527, F-44026 Nantes Cedex, France.

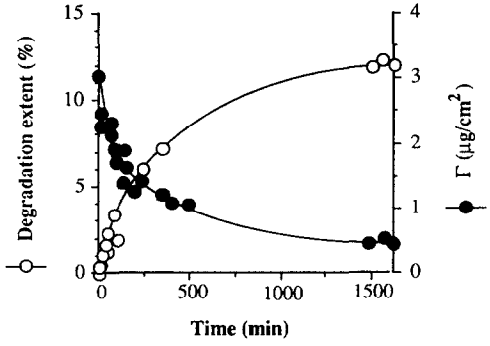


Fig. 1. Kinetics at 25° of the adsorption of porcine pancreatic alpha-amylase onto spherulites (●) and alpha-amylolysis (○).

(A–C) [18,19]. The largest domain (A, 330 residues) adopts a typical $(\alpha/\beta)_8$ barrel structure, within which domain B (68 residues) is inserted. The active site is found in a cleft located between domains A and B. Domain C (89 residues) forms a globular entity. Our purpose was first to identify the relationships between adsorption and catalysis, and secondly to characterise the mode of adsorption.

The adsorption experiment was carried out by centrifugation, leading to a time interval of ~ 4 min. Other techniques such as filtration cannot be used, because spherolites will clog the pores and disrupt the membrane. The adsorption of PPA-I onto the spherulites and the amylolysis kinetics are given in Fig. 1 Initially, the enzyme has a high affinity for starch crystallites as shown by its rapid adsorption onto the spherulite surface. The kinetics of adsorption are well described by a logarithmic relationship. The amount adsorbed ($\Gamma$) starts from a maximum (3.56 $\mu g/cm^2$) and drops to a plateau at 24 h (0.69 $\mu g/cm^2$). Degradation of starch crystallites is also of the logarithmic type and proceeds at a constantly decreasing rate [$\partial x/\partial t$ (%/min) = $2.44\%.t^{-1}$] before stopping at 24 h. The proportion of spherulites hydrolysed is then 12%. A small amount of enzyme was still adsorbed on the remaining spherolites, demonstrating ineffective adsorption. This feature has been observed for the specific binding of bacterial alpha-amylases on cross-linked potato starch [3,4]. Thus, adsorption precedes catalysis and is a prerequisite step for the hydrolysis of crystalline starch. Moreover, comparison of the curves shows that the decrease in the rate of hydrolysis is accompanied by desorption of PPA-I. In work on amylase/crystalline starch [5] or cellulase/crystalline cellulose [20–22] systems, the same tendencies of coupled desorption and eventual cessation of hydrolysis were observed. This behaviour was ascribed essentially to the formation of enzyme–product complexes, which cannot be adsorbed onto the spherulites and may be compared with the inhibiting properties of the products of hydrolysis in solution, such as maltose for alpha-amylase [23].

The products of hydrolysis were studied qualitatively and quantitatively at different stages of hydrolysis (Table I). After hydrolysis for 1 h, maltose ($G_2$,

TABLE I

Extent of hydrolysis and composition of oligosaccharides obtained by action of PPA-I onto crystalline starch as a function of time

| Time (h) | Extent of hydrolysis (%) | Total oligosaccharides (%) | | | | |
|---|---|---|---|---|---|---|
| | | $G_1$ | $G_2$ | $G_3$ | $G_4$ | $> G_5$ |
| 1 | 1.5 | 2.2 | 45.3 | 36.3 | 8.7 | 7.5 |
| 2 | 3.3 | 2.9 | 31.8 | 30.1 | 24.2 | 11 |
| 4 | 6.1 | 1.9 | 67.0 | 9.5 | 1.5 | 2.9 |
| 24 | 12 | 19.5 | 70.5 | 5.2 | 2.9 | 1.8 |

45.3%), maltotriose ($G_3$, 36.3%), and maltotetraose ($G_4$, 8.7%) were the main products. $G_3$ and $G_4$ were hydrolysed further and the proportions were $< 10\%$ after 24 h. At the same time, the concentrations of glucose ($G_1$) and $G_2$ increased and accounted for 19.5 and 70.5%, respectively, of the saccharides. The influence of $G_1$–$G_4$ upon adsorption was considered next. Fig. 2 shows the amount of enzyme adsorbed as a function of the saccharide : enzyme molar ratio (G : E). $G_1$ and $G_4$ had hardly any effect on the apparent adsorption equilibrium, whereas $G_2$, and particularly $G_3$, significatively decreased the amount of PPA-I adsorbed onto the spherulites for a G : E ratio of $> 1$. For a G : E ratio of 1000, $G_2$ and $G_3$ decreased the amount of enzyme adsorbed by 40 and 53%, respectively. This behaviour has been observed with *B. subtilis* alpha-amylase, although the effect was even more marked [5]. No adsorption was observed for $G_2$ : E and $G_3$ : E ratios of $> 10$. The smaller effect of $G_2$, compared to that of $G_3$, reflects a binding site specific for $G_2$ in pancreatic alpha-amylase [24]. This additional binding site would delay the effect of $G_2$ on adsorption, and thus explain the slight differences between porcine pancreatic and *B. subtilis* alpha-amylases. The resistance of crystalline starch to attack by amylase in vitro probably reflects the increasing amounts of enzyme–product complexes present in solution as the hydrolysis

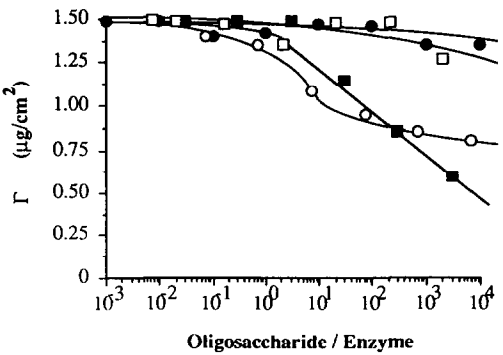


Fig. 2. Adsorption of porcine pancreatic alpha-amylase onto spherulites as a function of the oligosaccharide : enzyme molar ratio: ●, glucose; ○, maltose; ■, maltotriose; and □, maltotetraose.

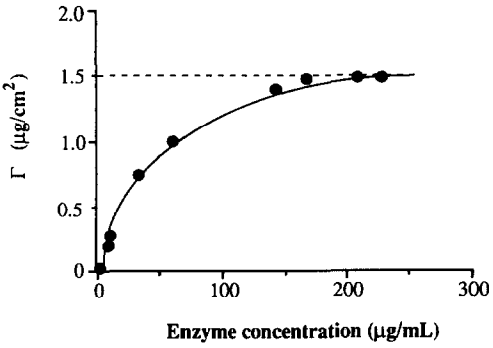


Fig. 3. Adsorption isotherm of alpha-amylase onto spherulites at 25°.

proceeds. Continuous removal or transformation (synergistic action of amyloglucosidase [25,26]) of the reaction products could allow hydrolysis of the resistant starch. It is uncertain that such a strong inhibition of adsorption exists in vivo, as the hydrolysis products are quickly absorbed through the enterocyte membrane.

The adsorption isotherm of PPA-I shown in Fig. 3 has a characteristic L-shape. The adsorption increases rapidly when the amount of PPA-I in solution at equilibrium increases from 0 to 150 $\mu$g/mL. At $>150$ $\mu$g/mL, the amount adsorbed reaches a plateau ($\Gamma_{max}$) of 1.49 $\mu$g/cm$^2$ and only 6.5% of the enzyme was adsorbed. This result is similar to that obtained with *B. subtilis* alpha-amylase [5] ($\Gamma_{max}$ 1.62 $\mu$g/cm$^2$), indicating that there is no strong dependence on the source of the alpha-amylase. Using the same calculations as for *B. subtilis* alpha-amylase [5], it follows that the hypothesis of monolayer adsorption for *B. subtilis* alpha-amylase might also apply to pancreatic alpha-amylase.

Adsorption onto the spherulites is a specific property of alpha-amylase, as shown by the non-adsorption of serum albumin [5]. However, in order to localise the region of the enzyme involved in the adsorption phenomenon, fragments of PPA-I corresponding to domains A/B and C were adsorbed onto crystalline starch. Both domains were adsorbed in the plateau region in amounts of 1.42 and 0.32 $\mu$g/cm$^2$, respectively. Domain A/B, which contains the active site of the enzyme, was adsorbed in amounts similar to that of PPA-I, but domain C was hardly adsorbed and probably in an unspecific manner (e.g., surface denaturation) because of the fragility of its secondary structure. This result suggests that pancreatic alpha-amylase is adsorbed through its active site, with specific interactions which are still to be determined. It also accords with the results obtained in the presence of oligosaccharides which compete with crystalline starch for the same binding region.

Desorption by dilution in water was also considered. Fig. 4 shows the percentage of enzyme desorbed as a function of time for two initial surface coverages of 50 and 100%. The amount of enzyme desorbed initially passes through a maximum (33 and 45%, respectively) before decreasing to 19 and 33% after 2 h. The

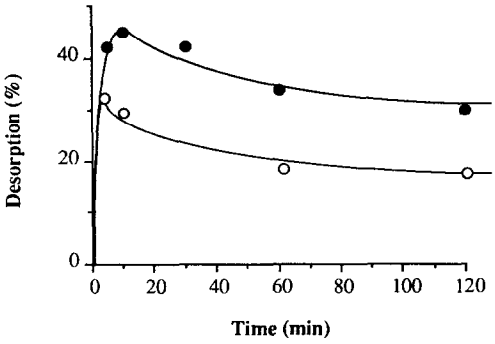


Fig. 4. Reversibility of adsorption of porcine pancreatic alpha-amylase onto spherulites as a function of initial surface coverage: ●, 100%; ○, 50%.

adsorption appears to be irreversible and dependent on the initial surface coverage. The slightly better desorption obtained at high surface coverage suggests a more labile mode of adsorption when the adsorption layer is compact. Some examples of quasi-irreversible adsorption (~ 10% desorption) have been observed for bacterial alpha-amylase [3]. However, Leloup et al. [5] found high percentages of reversibility (> 60% desorption) for the same enzyme species. These inconsistencies may be due to the differences in the substrates and enzyme sources. Desorbability also depends on the conditions of desorption. Rozie et al. [3] found almost total desorption when glycerol was added or the temperature was increased. We have found better desorptions when maltose or maltotriose was added to the medium. There is no unique explanation for the apparent irreversibility of adsorption. Low desorption properties are often associated with the presence of hydrophobic interactions of the sorbate and sorbant [27]. Henrissat et al. [28] noted the apparent irreversibility of adsorption for the cellulase–cellulose system and suggested a mode of adsorption that involved numerous interactions. These multiple enzyme–substrate interactions compensate for the lack of flexibility of the substrate, but are responsible for the strong adsorption of the enzyme. Similar behaviour may be suggested for the adsorption of PPA-I onto crystalline starch.

The influence of electrostatic effects on the mode of adsorption was studied by varying either the pH or the ionic strength ($I$). Tables II and III present the amounts of PPA-I adsorbed at the plateau ($\Gamma_{max}$) as a function of pH and ionic strength ($I$). Adsorption was a maximum in the pH range 6.0–6.5 ($1.78 < \Gamma_{max} < 1.83$ $\mu g/cm^2$). This pH zone also corresponds to the isoelectric point (p$I$) [18] of

TABLE II

Influence of the pH on the amount of PPA-I adsorbed ($\Gamma$) onto crystalline starch

| pH | 6.0 | 6.5 | 7.0 | 7.5 | 8.0 |
|---|---|---|---|---|---|
| $\Gamma$ ($\mu g/cm^2$) | 1.78 | 1.83 | 1.49 | 1.31 | 1.1 |

TABLE III

Influence of the ionic strength on the amount of PPA-I adsorbed ($\Gamma$) onto crystalline starch

| $I$ (mM) | 0.05 | 0.5 | 5 | 50 | 200 |
|---|---|---|---|---|---|
| $\Gamma$ ($\mu$g/cm$^2$) | 0.82 | 1.49 | 1.49 | 1.63 | 0.712 |

PPA-I, for which the enzyme is uncharged and has the highest intramolecular stability. Thus, the adsorption maximum in the pH range 6.0–6.5 results from both minimisation of enzyme–enzyme repulsive interactions and the lowest deformation of PPA-I upon adsorption. The adsorption was a maximum at pH 7.0 for the ionic strength range 5–50 mM ($1.49 < \Gamma_{max} < 1.63$ $\mu$g/cm$^2$). At $< 5$ mM, the charges on PPA-I, which are uncompensated, are responsible for repulsive interactions. At $> 50$ mM, the important solvation layer associated with PPA-I causes steric hindrance. In each situation, the amount of adsorbed PPA-I is decreased. For the intermediate range 5–50 mM, the charges on PPA-I are screened without a significant increase in hydrodynamic radius of the enzyme and the adsorption is a maximum. The electrostatic contribution determines mainly enzyme–enzyme interactions and influences the compactness of the adsorption layer; its involvement in the enzyme–substrate interactions is less clear.

The adsorption of the PPA-I is of the monolayer type, specific, and hardly reversible. The enzyme adsorbs through the domain A/B which contains the active site. Adsorption is necessary for the hydrolysis of crystalline starch, but is strongly inhibited by the reaction products (maltose and maltotriose). There are no marked differences in the adsorption behaviour of *B. subtilis* and pancreatic alpha-amylases, suggesting that these enzymes behave similarly towards solid starch. However, the overall process is far from being understood. In particular, there are still queries about the subsites, amino acids involved in adsorption, and the characteristics of the kinetic parameters. The determination of the adsorption free energy would allow useful comparisons to be made with the published data obtained for soluble substrates and may help in understanding the resistance of the crystalline starch. Recent reports [29,30] will help in the understanding of the phenomena.

## EXPERIMENTAL

Starch crystallites of the B-type, prepared by slow recrystallisation of (1 → 4)-$\alpha$-D-glucans and characterised as described [5], were spherical, monodisperse, highly crystalline, and had a smooth surface of specific area $A_s = 6.507$ cm$^2$/mg.

Isoamylase I (63 K) from porcine pancreas (PPA-I) was isolated, purified, and characterised as described [31]. Two fragments corresponding to the domains A/B (45 K) and C (18 K) of PPA-I were obtained by limited proteolysis using subtilisin [29,30].

*Kinetics of alpha-amylolysis and adsorption.*—Spherulites ($\sim 10$ mg) were added at 25° to the same mass of a PPA-I solution ($C_0 = 75$ $\mu$g/mL, 25 nkat/mg of

substrate) prepared in phosphate buffer (pH 7.0, 5 mM) containing $CaCl_2$ (0.25 mM) and NaCl (1.75 mM). The suspension was shaken continuously. Aliquots (1 mL) were withdrawn at intervals and centrifuged for 2 min at 2000*g*. The concentration of protein ($C$ in $\mu$g/mL) was determined by the method of Bradford [32] and the amount adsorbed $\Gamma$ ($\mu g/cm^2$) was calculated as follows:

$$\Gamma(\mu g/cm^2) = \frac{(C_0 - C)}{(mA_s)} \cdot V$$

where $m$ is the amount of spherulites (mg) and $V$ is the volume of the supernatant solution. The total soluble sugars were measured by the method of Tollier and Robin [33], and the extent of degradation was described by the percentage of sperulites hydrolysed. The oligosaccharide composition was analysed after inactivation of the enzyme (100°, 10 min) by HPLC on a column (25 × 0.46 cm) of $C_{18}$ silica gel (Merck) by elution with water at 15° at 0.8 mL/min. The column was calibrated using D-glucose to maltohexaose.

*Adsorption isotherm.*—Under the conditions described above, the adsorption isotherm for PPA-I was established with an initial enzyme concentration in the range 0–230 $\mu$g/mL. After adsorption for 2 h, an aliquot (1 mL) was centrifuged and the concentration of protein was determined. The amount of enzyme ($\Gamma$) adsorbed was calculated as stated above and expressed as a function of the concentration in solution at equilibrium ($C$). The adsorption of domains A/B and C of PPA-I was studied at 150 $\mu$g/mL in the same way.

*Specificity of adsorption.*—Spherulites (1 mg) were added to the enzyme solution (1 mL, 150 $\mu$g/mL) prepared in phosphate buffer (pH 7.0, 5 mM) containing $CaCl_2$ (0.25 mM), NaCl (1.75 mM), and 0–20 mg/mL of D-glucose, maltose, maltotriose, or maltotetraose. After adsorption for 2 h at 25°, the suspension was centrifuged and the concentration ($C$) of protein in the supernatant solution was determined. The amount of PPA-I adsorbed ($\Gamma$) was calculated as above and expressed as a function of the oligosaccharide : enzyme ratio (G : E).

*Reversibility of adsorption.*—Spherulites (2.5 mg) were suspended in the enzyme solution (2.5 mL, 75 and 150 $\mu$g/mL) prepared in phosphate buffer (pH 7.0, 5 mM) containing $CaCl_2$ (0.25 mM) and NaCl (0.75 mM). After adsorption for 2 h at 25°, the samples are centrifuged. The amount of PPA-I adsorbed ($\Gamma$) was determined as described above. The spherulites were then recovered with 2.5 mL of fresh buffer, aliquots (0.5 mL) were withdrawn at intervals during the following 2 h and centrifuged, the concentration of desorbed protein ($C_d$) in the supernatant solution was measured, and the percentage of enzyme reversibly adsorbed was calculated from the equation

$$R(\%) = \frac{C_d V}{\Gamma A} \cdot 100$$

where $V$ is the volume of fresh buffer added, $A$ is the total surface of the spherulites, and $R(\%)$ is given as a function of time.

*Effect of the pH and ionic strength.*—Spherulites (1 mg) were added to the enzyme solution (1 mL, 150 $\mu$g/mL) prepared in phosphate buffer (5 mM)

containing $CaCl_2$ (0.25 mM) and NaCl (1.75 mM) with pH ranging from 6.0 to 8.0. The amount of adsorbed enzyme ($\Gamma$) was determined as a function of pH as described above. The ionic strength ($I$) effect was studied similarly in the range 0.05–200 mM. $\Gamma$ was calculated as a function of $I$.

## REFERENCES


1 G.J. Walker and P.M. Hope, *Biochem. J.*, 86 (1963) 452–462.
2 Y. Kozaki and H. Inoue, *Hakko Kogaku Zasshi*, 62 (1984) 111–117.
3 H. Rozie, W. Sommers, A. Bonte, K. Van 't Riet, J. Visser, and F.M. Rombouts, *Biotechnol. Appl. Biochem.*, 13 (1991) 181–195.
4 H. Rozie, W. Sommers, K. Van 't Riet, F.M. Rombouts, and J. Visser, *Carbohydr. Polym.*, 15 (1991) 349–365.
5 V.M. Leloup, P. Colonna, and S.G. Ring, *Biotechnol. Bioeng.*, 38 (1991) 127–134.
6 A. Kieboom and H. Van Bekkum, in G.M. Van Beynum and J.A. Roels (Eds.), *Starch Conversion Technology*, Dekker, New York, 1985, pp. 263–334.
7 F.R.J. Bornet, A.-M. Fontvieille, S. Rizkalla, P. Colonna, A. Blayo, C. Mercier, and G. Slama, *Am. J. Clin. Nutr.*, 50 (1989) 315–323.
8 S.G. Ring, V.M. Leloup, and M.A. Stockham, U.K. Pat. 8926645.6 (1990).
9 N.-G. Asp, I. Björck, J. Holm, M. Nyman, and M. Siljeström, *Scand. J. Gastroenterol. Suppl.*, 129 (1986) 29–32.
10 C.S. Berry, *J. Cereal Sci.*, 4 (1986) 301–314.
11 J. Holm, I. Björck, S. Ostrowska, A.C. Eliasson, N.-G. Asp, K. Larsson, and I. Lundquist, *Staerke*, 35 (1983) 294–297.
12 T.F. Schweizer, S. Reimann, and P. Würsch, *Lebensm. Wiss. Technol.*, 21 (1988) 352–357.
13 T. Yamamoto, *Handbook of Amylases and related Enzymes: their Sources, Isolation Methods, Properties and Applications*, The Amylase Research Society of Japan, Osaka, 1988, pp. 18–75.
14 P. Colonna, A. Buleon, and V. Leloup, *Eur. J. Clin. Nutr.*, in press.
15 A. Imberty, H. Chanzy, S. Perez, A. Buleon, and V. Tran, *J. Mol. Biol.*, 201 (1988) 365–378.
16 A. Imberty and S. Perez, *Biopolymers*, 27 (1988) 1205–1221.
17 S.G. Ring, M.J. Miles, V.J. Morris, R. Turner, and P. Colonna, *Int. J. Biol. Macromol.*, 9 (1987) 158–160.
18 F. Payan, R. Haser, M. Pierrot, M. Frey, J.P. Astier, B. Abadie, E. Duee, and G. Buisson, *Acta Crystallogr., Sect. B.*, 36 (1980) 416–421.
19 G. Buisson, E. Duee, R. Haser, and F. Payan, *EMBO J.*, 6 (1987) 3909–3916.
20 A.O. Converse, R. Matsuno, M. Tanaka, and M. Tanaguchi, *Biotechnol Bioeng.*, 32 (1988) 38–45.
21 M. Tanaka, H. Nakamura, M. Tanaguchi, T. Morita, R. Matsuno, and T. Kamikubo, *Appl. Microbiol. Biotechnol.*, 23 (1986) 263–268.
22 C. David and R. Fornassier, *Macromolecules*, 19 (1986) 552–557.
23 P. Elodi, S. Mora, and M. Krysteva, *Eur. J. Biochem.*, 24 (1972) 577–582.
24 C. Seigner, E. Provdanov, and G. Marchis-Mouren, *Eur. J. Biochem.*, 148 (1985) 161–168.
25 J.L. Abe, F.W. Bergmann, K. Obata, and S. Hizukuri, *Appl. Microbiol. Biotechnol.*, 27 (1988) 447–450.
26 J.L. Abe, K. Nakajima, H. Nagano, and S. Hizukuri, *Carbohydr. Res.*, 175 (1988) 85–92.
27 W. Norde, *Adv. Colloid Interface Sci.*, 25 (1986) 267–340.
28 B. Henrissat, H. Driguez, C. Viet, and M. Schülein, *Biotechnol.*, 3 (1985) 722–726.
29 V. Desseaux, B. Svensson, F. Payan, R. Haser, and G. Marchis-Mouren, *Food Hydrocolloids*, 5 (1991) 209–213.
30 V. Desseaux, F. Payan, E.H. Ajandouz, B. Svensson, R. Haser, and G. Marchis-Mouren, *Biochim. Biophys. Acta*, in press.
31 C. Seigner, E. Provdanov, and G. Marchis-Mouren, *Biochim. Biophys. Acta*, 91 (1987) 200–209.
32 M.M. Bradford, *Anal. Biochem.*, 72 (1976) 248–254.
33 M.T. Tollier and J.P. Robin, *Ann. Technol. Agric.*, 28 (1979) 1–15.