p*-Nitrophenylmaltoheptaoside as Substrate for Cereal Amylases

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Amylases are hydrolytic enzymes that degrade starch; α amylases (α -1,4-D-glucan-glucanohydrolase, EC 3.2.1.1) are endoenzymes while β amylases (α -1,4-D-glucan-maltohydrolase, EC 3.2.1.2) are exoenzymes. As β amylases hydrolyse starch from the nonreducing end of the molecule, they cannot cleave the branch points (the α -1,6-glycosidic bonds); ' β limit dextrin' will be left as a product after complete β amylase attack on the starch substrate. That hydrolysis of starch affects the starch-iodine colour is commonly used for measuring the activity of both α and β amylases. The β limit dextrin, which also gives a colour reaction with iodine, is used as a specific substrate for determining α amylase activity in a solution which contains both α and β amylases¹. Unfortunately, these colorimetric methods are time-consuming and cannot be applied for following the reactions continuously. A commercially available synthetic substrate, *p*-nitrophenylmaltoheptaoside (Fig. 1), in combination with α glucosidase (α -D-glucoside glucohydrolase, EC

3.2.1.20), is suitable for determining α amylase activity in human serum and urine².

p-Nitrophenylmaltotrioside is hydrolyzed to *p*-nitrophenol and glucose very fast, whereas *p*-nitrophenylmaltotetraoside is split at a much lower rate, yielding negligible amount of products. Production of *p*-nitrophenol causes increase in the absorbance at 405 nm.

This method is continuous and rapid. Our purpose was to use *p*-nitrophenylmaltoheptaoside as a well defined substrate in our studies on α amylases from cereals, with emphasis on rye wheat (*Triticale*).

Fig. 1. *p*-Nitrophenylmaltoheptaoside.

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Experimental

Amylase-containing raw extracts from *Triticale* flour were prepared essentially as described by Weslake and Hill³. α Amylases from 600 g of grain were purified by *affinity chromatography* on cycloheptaamylose coupled to epoxy-activated Sepharose 6B according to the same authors³. Column dimensions were 16×140 mm; flow rate was 60 ml h⁻¹ and fraction volume 3 ml. Sodium acetate buffer (50 mM), pH 5.5, including 1 mM CaCl₂ was used. For final elution, the buffer was supplied with 7 mM cycloheptaamylose. For β amylases, *hydrophobic interaction chromatography* on phenyl Sepharose CL-4B⁴, followed by *chromatofocusing*⁵, pH 7.8-3.5, was used according to Pharmacia instruction manuals. Raw extract from 30 g of grain was used. For *hydrophobic interaction chromatography*, column dimensions were 16×150 mm; flow rate was 30 ml h⁻¹ and fraction volume 5 ml. Sodium acetate buffer (200 mM), pH 5.5, including 1 mM CaCl₂ worked as start buffer and the final solution was 100 % ethylene glycol. For *chromatofocusing*, column dimensions were 10×300 mm; flow rate was 24 ml h⁻¹; fraction volume 4 ml; sample volume 3.5 ml (peak III, Fig. 3); start buffer 25 mM imidazol/HCl, pH 7.4, and elution buffer Polybuffer 74, pH 4.0 (Pharmacia); final elution with 1 M NaCl. Isoelectric focusing (IEF) was performed on an LKB multiphor apparatus on polyacrylamide gels (C 5 %, T 1.5 %) with the pH gradient 3–10, generated by pharmalyte 3–10 according to the method described in the Pharmacia instruction manual⁶. The amylase isozymes were detected by staining for activity (zymogram technique): the IEF gel was firmly pressed against substrate-containing agar plates (1 %, w/v) including 0.2 % (w/v) of starch or 0.3 % (w/v) of β limit dextrin for 5 and 20 min, respectively. The starch and β limit dextrin plates were stained in a solution containing 0.088 % (w/v) of iodine and 4 % (w/v) of KI. β Limit dextrin was prepared by incubation for 24 h at 20 °C of 10 g of starch (soluble according to Zulkowsky, Merck) with 50 mg of barley β amylase (45 U/mg, Merck) in 0.2 M sodium acetate buffer, pH 4.7, containing 0.05 % toluene. The solution was then heated to 100 °C for inactivation of β amylase and concentrated by rotary evaporation to about 100 ml. About 30 ml were applied to a Sephadex G-25 column and eluted with water. Column dimen-

sions were 26×730 mm; flow rate was about 60 ml h⁻¹ and fraction volume 5 ml. The void volume fractions were freeze-dried and used as β limit dextrin.

Amylase activity with β limit dextrin as substrate was determined according to the method described by Briggs¹, modified by Olered and Jönsson⁷. Activity using *p*-nitrophenylmaltoheptaoside as substrate was measured according to Rauscher *et al.*² (α amylase PNP kit from Boehringer, Mannheim, W. Germany), with some modification: 50 mM imidazole buffer (pH 7.0), including 1 mM CaCl₂, replaced the phosphate buffer. Activity was determined for both α and β amylase at various concentrations of the synthetic substrate. By least square fits of the data to a hyperbola, according to Cleland⁸, the apparent Michaelis constant, K_m , and calculated enzymic activity at infinite concentration of the substrate concentrate, V_m , were estimated.

Results

Affinity chromatography on cycloheptaamylose coupled to epoxy-activated Sepharose 6B of the flour extract resulted in two peaks (Fig. 2); the first one contained material which did not fix to the column matrix and the second was eluted with cycloheptaamylose. The material in peak I showed high amylase activity with *p*-nitrophenylmaltoheptaoside as substrate; a much lower

Fig. 2. Affinity chromatography on cycloheptaamylose coupled to epoxy-activated sepharose 6B of a salt extract from flour of *Triticale*.

Table 1. Tests for protein (A_{280}) and enzymic activity in the material eluted in the peak fraction I–IV in Figs. 2–4. With 5 mM *p*-nitrophenylmaltoheptaoside as substrate, the linear increase in absorbance at 405 nm with time, occurring 3 min after mixing of substrate and enzyme, was taken as activity (ΔA_{405}) and $\Delta A_{405}/A_{280}$ as specific activity.

Peak	A_{280}	Activity/ $\text{ml}^{-1}\text{min}^{-1}$	Specific activity/ $10^2\text{ml}^{-1}\text{min}^{-1}$	K_m / mM
I	17	950	0.6	
II ^a	0.11	27	2.4	9
III ^b	1.1	15	0.1	
IV ^b	0.20	56	2.8	0.09

^aAfter gel filtration on Bio Gel P-6DG and concentration.

^bAfter gel filtration on Sephadex G-25 concentration.

Fig. 3. Hydrophobic interaction chromatography on phenyl-Sepharose CL-4B of a salt extract from flour of *Triticale*.

Fig. 4. Fractionation of material from peak III in Fig. 3 by chromatofocusing in pH region 7.8–3.5.

activity was found in peak II (Table 1). α Amylase activity with β limit dextrin as substrate was found only in peak II.

Fig. 3 shows the chromatogram obtained after separation of the flour extract on phenyl Sepharose. Most of the peak fractions were active with *p*-nitrophenylmaltoheptaoside as substrate. To purify β amylase, the material in peak III was further separated by chromatofocusing (see Fig. 4). Activity on *p*-nitrophenylmaltoheptaoside was mainly detected in peak IV. In tests for α amylase activity with substrate β limit dextrin, no activity was obtained in the peak fraction IV.

The materials from peak II (Fig. 2) and peak IV (Fig. 4) were analysed by isoelectric focusing. When the zymogram technique using starch was utilized for amylase identification, the proteins in peak II developed a pattern characteristic⁹ for α amylase isozymes; for peak IV the pattern was typical⁹ for β amylase isozymes. There was no evidence suggesting peaks II or IV to be contaminated with β or α amylase, respectively. The zymogram technique with β limit dextrin showed the characteristic bands for α amylase in the material from peak II. No protein bands arising from peak IV were stained with β limit dextrin. The results clearly show that separated α amylase (Fig. 2, peak II) and β amylase (Fig. 4, peak IV) fractions were isolated.

The K_m values for *p*-nitrophenylmaltoheptaoside when acting as substrate for α amylase and β amylase were found to be 9 mM and 0.09 mM, respectively. The K_m value of human serum α amylase is 0.12 mM². At the substrate concentration used for testing the activity presented in Table 1, β amylase from cereals and α amylase from human serum should show optimal levels of activity. The estimated maximal activity (V_m) for the material in peak IV was close to (2% higher) the given specific activity (Table 1). For α amylase (peak II fraction, V_m was $6.9 \cdot 10^{-2} \text{ ml}^{-1} \text{ min}^{-1}$ instead of $2.4 \cdot 10^{-2} \text{ ml}^{-1} \text{ min}^{-1}$ as given in Table 1. Thus the apparent catalytic rate constant is higher for α amylase than for β amylase. That the K_m value is two orders of magnitude higher for cereal α amylase than for β amylase can be used for separately measuring cereal β amylase activity, assuming suitable substrate concentrations are selected. If the activity reported in Table 1 were run at 0.1 mM instead of 5 mM of substrate the corresponding specific activity (see legends to Table 1) should be about half the value (or

$1.5 \cdot 10^{-2} \text{ ml}^{-1} \text{ min}^{-1}$) given for β amylase (peak IV). The specific activity of α amylase under corresponding conditions is about 5 % of the value of β amylase. Such proportions between the two activities should be found in an enzyme preparation containing equivalent amounts of α and β amylase. In cereals, the β amylases appear to dominate the α amylases. It is reasonable to suggest that the amounts of the former are more than twice the amounts of the latter. Under these conditions, α amylase has only a marginal effect on β amylase activity and *p*-nitrophenylmaltoheptaoside can be used as a specific substrate for β amylase. For tests of α amylase activity, a much higher substrate concentration is needed, and β amylase contamination must not exist. If β amylase is present the activity has to be measured at two substrate concentrations.

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