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Partial Characterization of a Thermostable Anthocyanin-β-Glycosidase from *Aspergillus niger*

Hans Blom

Norwegian Food Research Institute,
PO Box 50, N-1432 Aas-NLH, Norway

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ABSTRACT

Anthocyanin-β-glycosidase (anthocyanase) from Aspergillus niger *has been partly purified and shown to catalyse a lytic reaction upon the β-glycosidic bond of anthocyanins. The molecular weight as estimated from high performance liquid chromatography and gel chromatography on Ultrogel AcA-34 is in the range* $(370 \pm 30) \times 10^3$. *SDS electrophoresis indicates a tetrameric composition. Isoelectric point is located at pH 4.*

INTRODUCTION

Anthocyanins are among the most widespread pigments in nature. They give the red, purple and blue colour to berries, and their contribution is no doubt one of the most important factors for the visual impression of jams and soft drinks.

Chemically, the anthocyanins are composed of a flavylium ion (Timberlake & Bridle, 1975) glycosylated with a β-glycosidic bond (Ribérau-Gayon, 1972). The different colour nuances are obtained through hydroxylation and methylation of the flavylium ion and through the combination of other factors such as pH, metal ions, etc., in the natural environment of the anthocyanins. If the β-glycosidic bond is broken the pigments become unstable and are easily converted to brown or colourless compounds (Eskin, 1979).

Enzymes capable of degrading anthocyanins are reported (Huang, 1955; Pifferi *et al.*, 1979). Mechter demonstrated this fact as far back as

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1954 (personal communication with Huang, 1955). Today, anthocyanase is commercially available, mainly for use in the decoloration of wine.

In recent years, many artificial colouring agents have been banned as food additives due to their potential genotoxicity. Thus, the producer is increasingly more dependent on the naturally occurring pigments present in the fruits and berries. This problem has the greatest technological interest when considering production based on raw materials which are low in anthocyanin content. Thus, it seems reasonable to focus the research on anthocyanase producing non-pathogenic microorganisms to the thawing phase. That is when the freeze-damaged berries are exposed to the action of these enzymes.

The aim of the present study was to characterize one of the enzymes that decolorize anthocyanins; thus, the significance of these enzymes for colour stability in the storing of fresh berries and during the thawing of frozen berries can be established.

MATERIALS AND METHODS

Anthocyanase source

The anthocyanin-β-glycosidase was separated and purified from a pectolytic enzyme preparation named Pectinol D, production year 1976 (Röhm GmbH, Darmstadt, West Germany), an enzyme preparation made from a thermophilic strain of *Aspergillus niger* (personal communication with E. Grampp, Röhm GmbH, Darmstadt, West Germany).

Anthocyanin and pectin solution

Crude anthocyanin and pectin preparations were made from frozen strawberries (Senga sengana) which were homogenized with equal volumes of 0·1 M citrate buffer, pH 4·0, thus obtaining anthocyanins in a solution of about 0·2% pectin.

Preliminary assay for anthocyanase and pectinase activity

Efforts were made to separate anthocyanase activity from the pectolytic activity. A rapid method for a preliminary check on both enzymes in one operation was developed.

Anthocyanin and pectin solution (2 ml) was mixed with 0·5 ml of the sample to be tested and incubated for 2 h at 50 °C in a shaking waterbath. Pectinase activity was measured as a decrease in the viscosity of the solution. The reaction was stopped by adding 2·5 ml methanol made 0·1 N with concentrated HCl, thus precipitating the pectin and increasing the intensity of the pigment colour. The results of the test could be judged visually or, in certain cases, by absorbance measurements at 520 nm for anthocyanase activity.

Standard assay for anthocyanse activity

Anthocyanase activity was confirmed by separating glycones from aglycones by means of high performance liquid chromatography (Spectra Physics, Model 3500B). The chromatography was performed using an ODS-Hypersil column and acetic acid:water (1:7) as eluent with methanol increasing from 0 to 25 % in 8 min as gradient (Blom, 1981).

Standard assay for pectinase activity

Pectolytic activity was determined by monitoring increase in reducing sugar according to the method described by Nelson (1944), using pectate as substrate.

Molecular weight determinations

The molecular weight of the anthocyanase was estimated by gel filtration on Ultrogel AcA-34 (1·6 × 90 cm) in 0·1 M citrate buffer, pH 4·0, monitoring protein concentration at 280 nm.

The molecular weight was also estimated by HPLC (Spectra Physics) gel filtration on column: Bio-Sil, TSK-125 (300 × 7·5 mm). The eluent used was 0·065 M phosphate buffer, pH 6·8, in 0·1 M KCl. Elution was performed at a flow of 1 ml min^{-1} and with detection at 254 nm.

Sodium dodecyl sulphate gel electrophoresis was carried out according to the slab gel technique described by Laemmli & Favre (1973).

Isoelectric focusing

The isoelectric point of the anthocyanase was determined according to the procedure described by LKB-Products AB (1979), Bromma, Sweden.

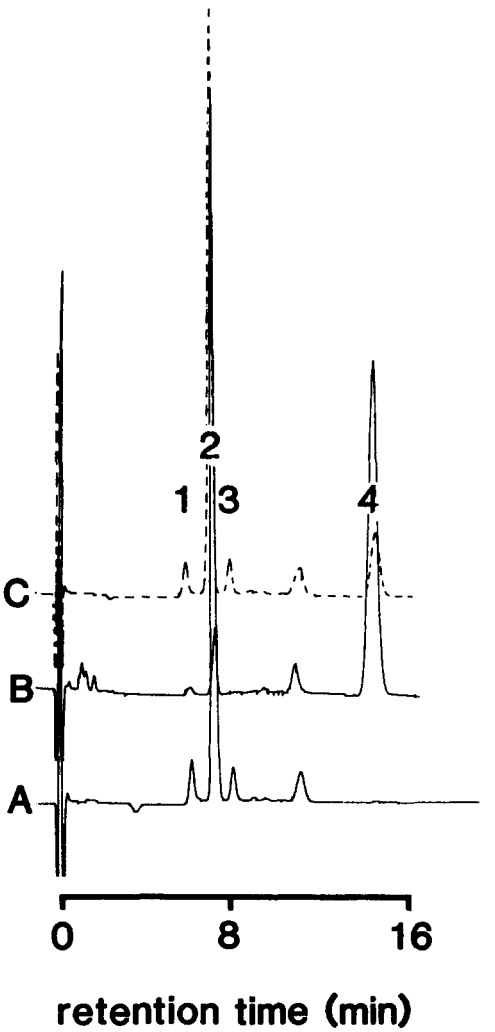

Fig. 1. HPLC of strawberry anthocyanins. Peaks labelled as follows: pelargonidine-3,5-diglucoside (1), pelargonidine-3-glucoside (2), cyanidine-3-glucoside (3) and pelargonidine (4). Chromatograms: A, of native strawberry anthocyanins; B, after 30 min acid hydrolysis (1 N HCl, 80 °C) of (A); C, after 30 min enzyme treatment (0·1 % Pectinol D, 50 °C) of (A).

Pooling the fractions from gel filtration on Ultrogel AcA-34 showing anthocyanase but no pectolytic activity, gave results from the molecular weight estimations as presented in Fig. 2.

The Ultrogel AcA-34 method gives an estimated molecular weight in the region $(350-400) \times 10^3$ and the experiments with HPLC $(370 \pm 30) \times 10^3$, but with the addition of two peaks at molecular weights 5000 and 2000 respectively. SDS electrophoresis repeatedly shows two protein bands corresponding to molecular weights of $(105 \pm 10) \times 10^3$ and $(60 \pm 3) \times 10^3$.

Isoelectric focusing experiments were first run in ampholine, at pH 3·5–10. Anthocyanase activity was found in the fractions at pH 3·78–4·24. The experiment was also carried out in a gradient of ampholine at pH 2·5–4·0, thus obtaining a less steep gradient. The anthocyanase activity was focused in only one fraction with pH 4·05, indicating a pI very close to 4.

DISCUSSION

The chromatographic patterns obtained after acid hydrolysis and enzymatic treatment of the anthocyanins both give a clear indication of a lytic action on the β-glycosidic bond. The enzyme could then be classified as a lyase (splits without the addition of water), or a hydrolase (splits with the addition of water). From the literature describing the monitoring of enzyme activity based on the analysis of the increase in reducing sugars (Huang, 1955) a hydrolytic mode of action is to be expected. The name of the enzyme should, consequently, be anthocyanin-β-glycosidase, and not the unspecific name anthocyanase, which includes the whole class of enzymes with various hydrolytic actions on the anthocyanin molecule.

It is evident from the molecular weight determinations that we are dealing with a large protein. Gel filtration on Ultrogel AcA-34 results in an elution volume very close to the void volume. The exclusion limit of Ultrogel AcA-34 is indicated at a molecular weight of 350 000 for globular proteins. Elution volumes of known protein standards give an estimate very close to this value, although it should be emphasized that estimating molecular weights of proteins of this size are encumbered with great inaccuracy, mainly due to imperfect globularity. High performance liquid chromatography on a molecular sieve column repeatedly gives one peak with an elution time corresponding to a molecular weight of $(370 \pm 30) \times$

10^3. In addition, two peaks at molecular weights of 5000 and 2000 appear, even though the sample has been rechromatographed on Ultrogel AcA-34. Injection of 0·1 M citrate buffer, pH 4·0, into the Bio-Sil TSK-125 column results in two peaks at molecular weights 5000 and 2000. This is due to the absorbance of citric acid at 254 nm, combined with an ion exchange effect of the column.

The results from the SDS slab gel electrophoresis could be interpreted as if we have two possible structural alternatives:

1. The overall structure could be that of a tetramer composed of subunits with molecular weights of about 100 000; i.e. a native protein of molecular weight about 400 000.
2. The enzyme could be a double dimer, i.e. consisting of two subunits with molecular weights close to 100 000 plus two subunits with molecular weights close to 60 000. Consequently, the total molecular weight would be about 320 000. However, the colour intensity of the band corresponding to molecular weight 60 000 is very low compared to that of the band corresponding to molecular weight 100 000, leaving an impression that the 60 000 band is a result of a degradation of the 100 000 band.

The isoelectric point at pH 4 is very close to the reported pH optimum of the enzyme which is indicated at pH 3·9 (Okada *et al.*, 1968). Taking into consideration the size of the enzyme molecule one can very well imagine that the molecule itself could be adequately polarized with respect to charges to exhibit maximal activity at this pH. However, it should be emphasized that the assay of the enzyme activity has so far been based on the rate of pigment decolorization. This includes the conversion of aglycones to colourless pseudobases as well, a reaction with a velocity that increases with increasing pH. At pH 4 the enzyme reaction product concentration will be rather small, thereby speeding up the reaction. This is a fact that should be taken into account when estimating the pH optimum for this particular enzyme.

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