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The Separation of Pectinlyase from β-Glucosidase in a Commercial Preparation

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Abstract: This paper describes a new, simple and inexpensive procedure for separating pectinlyase (PL, EC 4.2.2.3) from β-glucosidase (β-glu, EC 3.2.1.21), both of which have potential for use in the beverage processing industry. The method described here, which entails the treatment of crude preparations with bentonite (4% (w/v)) and the acidification of the resulting supernatant to pH 3·5, leads to the production of two enzymic solutions which contain PL and β-glu, respectively. In both solutions the amount of brown pigment is considerably less than in the crude mixture, and partial purification from extraneous proteins is also achieved.

Key words: separation, purification, enzymes, pectinlyase, β-glucosidase.

1 INTRODUCTION

The study of β-glucosidase (β-glu, EC 3.2.1.21), and of pectic enzymes in general, is of special interest because of the potential use of these enzymes in the food processing industry and in industrial biotechnology. For example, β-glucosidase could be employed in the processing of beverages such as wine and blood-orange juice. In fact, hydrolysis of anthocyanins into anthocyanidins and sugars could be used to obtain rosé wines richer in extracts and of the desired shade of red.[1–4] Moreover, the hydrolysis of the anthocyanins and the removal of the insoluble aglycons could prevent the discoloration of blood-orange juice during pasteurization. Studies[5,6] have shown that this enzyme may also have potential uses in the production of white wines which, because of the hydrolysis of glycoside monoterpenes, are richer in aroma.

Pectic enzymes, on the other hand, are commonly used in fruit juice processing.[7] In fact, the enzymic treatment of crude pulp and fruit juice essentially increases the quantity of extracted juice, lowers the processing time and the viscosity (thus making juice concentration processes easier), and reduces the turbidity in the preparation of clear fruit juice. The pectic enzymes most widely used in fruit juice processing include pectinesterase (PE, EC 3.1.1.11) and endo-polygalacturonase (PG, EC 3.2.1.15), both of which are necessary because PG is capable of depolymerizing pectins only after partial de-esterification by PE. Endo-pectinlyase (PL, EC 4.2.2.3), on the other hand, is capable of directly depolymerizing highly esterified pectins,[8] thus avoiding some of the problems which are closely associated with the use of PE. These include the release of methanol and the precipitation of the pectin, which has been de-esterified with endogenous Ca^{2+}, in the system.[9] The purpose of this study was to separate PL from β-glu in a commercial enzyme preparation Cytolase PCL 5 (Genecor). This preparation, which is quite inexpensive and readily available on the market, whilst possessing high pectinlyasic and β-glucosidasic activities, contains elevated amounts of impurities such as extraneous enzymes, proteins, mucilage and melanoidins which make it unsuitable for use in specific applications in the food processing industry. Separation of the two enzymes is necessary in order to avoid undesired juice modifications. Purification of these enzymes by traditional methods (chromatography)[10–14] would make the process too expensive and thus unsuitable for industrial applications. The present paper describes a novel, simple and economical separation method.

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2 MATERIALS AND METHODS

2.1 Materials

The enzyme preparations employed were: Cytolase PCL5 lot 45 (Genecor), Pectinol D5L (Rohm), Ultrazim 100 (Ciba-Geigy), and Rohapect (Rohm). Bentonite (BDH) was employed for purification. The following reagents were used as substrates: oenocyanin (Enocanossa, Reggio Emilia), 4-nitrophenyl-β-D-glucopyranoside (pNPG Aldrich) and apple pectin (Roth) esterified up to 98% according to the method given by Kohn *et al.*[15] Trichloroacetic acid (C. Erba), Coomassie Blue G 250 (Aldrich) and bovine albumin (Sigma) were employed for protein determination.

2.2 Methods

2.2.1 Substrate preparation

Monomer anthocyanins were extracted from commercial solid oenocyanin according to the procedure described previously.[16]

2.2.2 Activity tests

2.2.2.1 Pectinlyase. One cm^3 of 0·07 mol dm^{-3} citrate–phosphate (C–P) buffer at pH 5·0 and 1 cm^3 of an appropriately diluted enzyme solution were added to 2 cm^3 of a 1% (w/v) solution of 98% esterified pectin dissolved in the same buffer and incubated at 25°C, under stirring. After 1 min, 2 cm^3 of 0·5 mol dm^{-3} H_2SO_4 were added in order to stop the reaction. The blank was obtained by reversing the order of the addition of reagents to the pectin solution, i.e. the acid was added first, followed by the enzyme. The sample was measured against the blank at 235 nm; observed increases in absorbance were caused by the double conjugate bond of the Δ4:5 unsaturated uronide formed during the reaction. One unit of enzyme is defined as the amount which catalyses an increase of 0·555 in adsorbance at 235 nm in 1 min at 25°C and at optimal pH and ionic strength conditions.[17]

2.2.2.2 β-Glucosidase

(a) *Activity tests for β-glucosidase employing a pool of 3-monoglucoside anthocyanins.* One cm^3 of 0·1 mol dm^{-3} C–P buffer at pH 3·5 and 2 cm^3 of an appropriately diluted enzyme solution (PCL 5) were added to 1 cm^3 of a 0·15% (w/v) polyphenol-free solution of solid oenocyanin (Enocanossa) dissolved in 0·001 mol dm^{-3} HCl. The mixture was incubated for 30 min at 26°C. Two cm^3 of 0·1 mol dm^{-3} HCl were then added and absorbance was measured at 525 nm after 15 min. The activity unit (AU), defined as $\Delta A = 0{\cdot}028\ min^{-1}$ at 25°C, is equal to the degradation of 1 μmol of anthocyanin in a 1 dm^3 reaction volume. AU = (initial absorbance − final adsorbance) × $6 \times f/28 \times 30 \times 2$, *where* f *is the dilution factor and* 28 000 $dm^3\ mol^{-1}\ cm^{-1}$ is the molar extinction coefficient of anthocyanin in 0·1 mol dm^{-3} HCl.[18]

(b) *Activity tests for β-glucosidase employing pNPG.* Activity on pNPG was determined by adding (0·1 cm^3 of enzyme to 0·9 cm^3 of 0·1 mol dm^{-3} C–P buffer (pH 3·5) containing pNPG (final concentration $0{\cdot}5 \times 10^{-2}$ mol dm^{-3}). The reaction was carried out at 26°C for 1 min under stirring, after which it was stopped by adding 2 cm^3 of 1 mol dm^{-3} Na_2CO_3. The amount of *p*-nitrophenol released was measured on the spectrophotometer at 400 nm. The molar extinction coefficient employed was 18 300 $dm^3\ mol^{-1}\ cm^{-1}$. One activity unit of β-glucosidase is defined as the amount of enzyme required for the hydrolysis of 1 μmol of substrate (pNPG) per minute.[19,20]

2.2.3 Protein determination

Protein concentration was determined after precipitation in 7% (w/v) trichloroacetic acid by means of Coomassie Blue G250 and by employing bovine serum albumin as a standard.[21]

2.2.4 Separation of pectinlyase from β-glucosidase

Fifty cm^3 of enzyme solution, first diluted with 30·0 cm^3 of 0·1 mol dm^{-3} C–P buffer at pH 3·0, 4·0, 5·0 or 6·0, and then brought to the desired pH value by the addition of 1 mol dm^{-3} HCl or 1 mol dm^{-3} NaOH, were left in contact with 2·0 g of bentonite which had previously been swollen in 20·0 cm^3 of the same C–P buffer. Adsorption was carried out under stirring for 2·5 h at 25°C. The mixture was centrifuged at 1600 *g* at 4°C for 15 min, after which the supernatant (A) was acidified with 1 mol dm^{-3} HCl to pH 3·0–4·0, diluted twice with distilled water, allowed to stand overnight at 4°C and finally centrifuged at 3000 *g* at 4°C for 50 min.[22] Pectinlyase remained in the supernatant fluid (B), while β-glucosidase was precipitated together with mucilages (precipitate B).

For the recovery of β-glucosidase from the gelatinous precipitate a number of parameters were taken into account, e.g. the washing of the precipitate in water or in the buffer in the presence of salt, the nature of the salt (KCl, NaCl), salt concentration (0·25–4 mol dm^{-3}) and the pH value (2·5–7·5). The suspension thus obtained was then centrifuged at 3000 *g* and at 4°C for 20 min. The supernatant fluid (C) which contained β-glucosidase was passed through an ultrafiltration membrane with a molecular weight cut-off of 50 000 (XM-50 Amicon) (Fig. 1).

In order to optimize the purification and the separation of the two enzymes, the following parameters were taken into account: recovery (%) of enzymic activity in the supernatant (% recovery), increase in specific activity (purification) and drop in absorbance at 420 nm (A_{420}). These parameters were defined as follows:

Recovery % = (final activity/initial activity) × 100

Purification = (specific final activity/specific initial activity

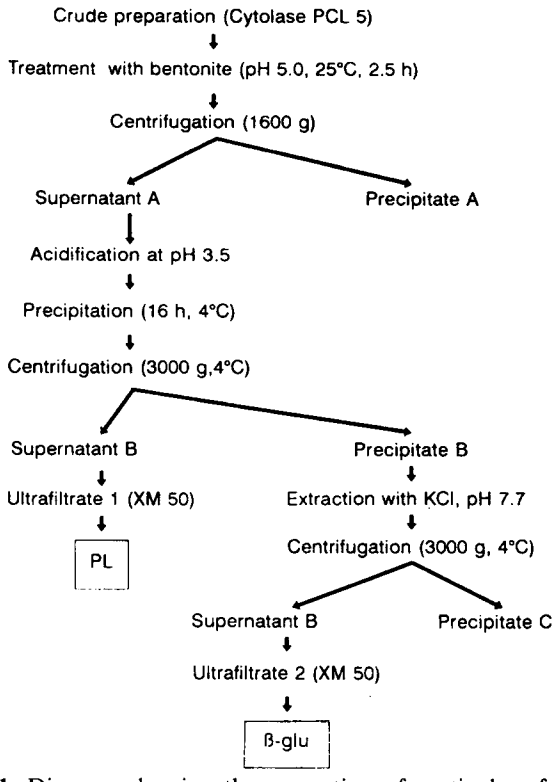

Fig. 1. Diagram showing the separation of pectinylase from β-glucosidase.

A_{420} = ((initial Abs_{420} − final Abs_{420})/initial Abs_{420}) × 100: this parameter was taken as an indicator of the presence of brown compounds (melanoidins).

3 RESULTS AND DISCUSSION

Of the various commercial preparations tested for PL and β-glu activity (Table 1), Cytolase PCL5 was chosen because of its high enzymic activity and specific activity. The treatment of this enzyme preparation with bentonite at pH 5·0 (Fig. 2) gave the best results in terms of retention of enzyme activity (100% for PL and 94% for β-glu) in the supernatant fluid. The high recovery of enzymic activity reported at pH values ranging from 4 to 6 probably reflects the fact that enzymes with a net charge were not capable of interacting with bentonite. At pH 3·0, on the other hand, the recovery of activity of the two enzymes in the supernatant fluid decreased since bentonite behaved as a cation exchanger with respect to the PL and the β-glu which were positively charged at this pH value (pI_{PL} about 3·7; $pI_{\beta\text{-glu}}$ about 3·9). The optimum conditions for treatment with bentonite were therefore found to be as follows: pH 5·0, enzyme diluted ratio 1:2 and contact time 2·5 h at room temperature (approx. 25°C).

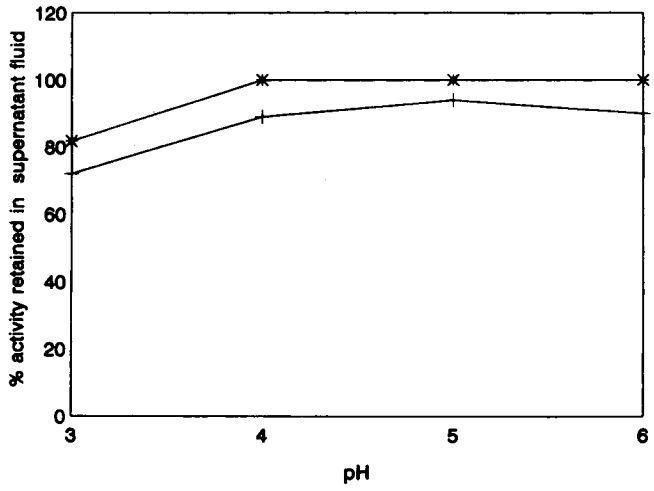

Fig. 2. PL (*) and β-glu (+) activity retained in the supernatant fluid following PCL5 treatment with bentonite (4% (w/v)) as a function of pH, at 25°C and 2·5 h.

Separation of the two enzymes was achieved after acid precipitation (pH = 3·5) from the supernatant fluid of the PCL5 preparation which had been previously treated with bentonite. At pH values below 3·0, even if the precipitate was obtained, a partial inactivation of the β-glu was observed, while at pH values above 4·0 there was only a small amount of precipitate. Following this treatment, 100% of the PL remained in solution while

TABLE 1
Characteristics of the Commercial Preparations Containing PL and β-Glu. The Substrate Employed for β-Glucosidase Consists of a Pool of Anthocyanins

	PL activity ($\mu mol\ min^{-1}\ cm^{-3}$)	*β-glu activity* ($\mu mol\ min^{-1}\ cm^{-3}$)	*Proteins* ($mg\ cm^{-3}$)	*Specific activity* ($\mu mol\ mg^{-1}$)	
				PL	*β-glu*
Liquid products					
Cytolase PCL5	120	1.5	33	3.6	0.045
Pectinol D5L	22·5	0·35	12·21	1·8	0·028
Solid products					
Ultrazim 100	0·066	0·002	0·058	1·1	0·034
Rohapect D5S	0·026	0	0·091	0·28	0

TABLE 2
PL and β-Glu Activity Reported in Supernatant B and in Precipitate B after Acidification and Centrifugation of Supernatant A obtained from the Crude Preparation PCL5 after Treatment with Bentonite at pH 5·0 and 25°C

	β-glu activity (%)	*PL activity* (%)
Supernatant B	26·5	100
Precipitate B	73·5	0

TABLE 3
Extraction of β-Glucosidase from Precipitate B Diluted Twice, as a Function of pH and of Salt Concentration

pH	*NaCl* (*mol dm*$^{-3}$)	*β-glu supernatant activity* (%)
2·5	0·25	0
	0·50	0
	1·00	0
4·0	0·25	0
	0·50	0
	1·0	0
7·7	1·0	56
	2·0	56
	3·0	56
	4·0	58
	5·0	51

73·5% of the β-glucosidase was recovered as a precipitate (Table 2). At acid pH values (2·5–4·0) β-glucosidase strongly interacted with mucilage and melanoidins. Despite the increase in ionic strength (0·25–1·0 mol dm^{-3} NaCl), this interaction prevented the extraction of the enzyme from the precipitate which had been twice resuspended in water (Table 3). In fact, at an average acid pH value (2·5 and 4·0), interactions may occur between negatively charged groups of pectins and melanoidins or hydrogen bonds can be formed between the oxydryl phenolic groups of the same melanoidins and the more or less protonated NH_2 groups of the enzyme to be separated. The interactions between β-glucosidase and the above mentioned polymers decrease at alkaline pH values. In fact, at pH 7·7 and with increasing ionic strength (1·0–5·0 mol dm^{-3}), an increase in enzyme extraction was observed. At an optimum NaCl concentration (4 mol dm^{-3}) 58% of β-glucosidase activity was extracted (Table 3). To achieve greater enzyme recovery, additional parameters were investigated such as the use of water or of 0·1 mol dm^{-3} C–P buffer and the nature of the salt during the washing of the precipitate. Table 4 shows that the presence of the buffer did not affect enzyme yield while that of KCl improved recovery of the enzymic activity. The method described here, as well as making

TABLE 4
Effects of Buffer and Salt on the Extraction of β-Glucosidase from the Precipitate B Obtained at pH 3·5 and 4°C after 16 h (see Fig. 1)

Extraction solvent	*Salt* (*4 mol dm*$^{-3}$)	*β-glu supernatant activity* (%) *from precipitate B*
Aqueous solution, pH 7·7[a]	NaCl	73·6
	KCl	82
C–P buffer, 0·1 mol dm^{-3}, pH 7·7[a]	NaCl	74

[a] Precipitate dilution factor: 1:8.

separation of the enzymes possible, also offers additional advantages such as the reduction of absorbance at 420 nm (this parameter being taken as the indicator of the presence of brown pigments or melanoidins) as well as that of extraneous proteins. In fact, treatment of PCL5 with bentonite permits the almost total recovery of PL and β-glu activity in the supernatant fluid and a 6·1% reduction of absorbance at 420 nm (Table 5). No increase in specific activity and the consequent purification factor were, however, achieved.

The acidification and precipitation processes lead to the formation of two ultrafiltrates. Ultrafiltrate 1, as well as containing three times as much PL as the initial preparation, also exhibited a reduction of 34·5% in absorbance at 420 nm. Ultrafiltrate 2, obtained after extraction with KCl, contained β-glu purified five times with respect to the crude mixture. It was characterized by a considerable reduction in brown pigments, the reduction in absorbance at 420 nm being 78·5% (Table 5).

4 CONCLUSION

The methods employed for protein purification are generally time-consuming and expensive and are therefore not suitable where the cost of enzymes has to be low such as in the food processing industry. On the other hand, commercial enzymic preparations containing high amounts of enzymes are not employed in food treatment as they may contain high amounts of extraneous substances which makes them liable to restrictions prescribed by law or may give rise to problems associated with undesired collateral activities (e.g. phenolasis, peroxidases, etc.). Yet, in some cases these commercial preparations could be purified by means of simple and inexpensive techniques that exploit only the chemical and physical properties of the desired proteins as well as those of the adsorbent. The adsorbent used in this study (bentonite) is readily available, inexpensive and non-toxic, and the separation method is fast and simple to carry out, yielding two ultrafiltered enzyme solutions.

TABLE 5

Separation and Purification of PL and β-Glu after Treatment with Bentonite (Supernatant A), Precipitation of the Supernatant at pH 3·5 and Ultrafiltration (Ultrafiltrate 1), Extraction of the Precipitate with 4 mol dm^{-3} KCl and Ultrafiltration (Ultrafiltrate 2). The Substrate Employed for β-Glucosidase is 4-Nitrophenyl-β-glucopyranoside

	Total activity (*μmol* min^{-1})		*Proteins* (*mg*)	*Specific activity* (*μmol* mg^{-1})		*Puriciation factor*		% *Reduction* ΔA_{420}
	β-glu	*PL*		*β-glu*	*PL*	*β-glu*	*PL*	
Crude preparation Cytolase PCL5	2120	6000	1650	1·28	3·6	—	—	—
Supernatant A	1993	6000	1390	1·43	4·31	1·1	1·2	6·1
Ultrafiltrate 1	502	5800	493	—	12	—	3	34·5
Ultrafiltrate 2	1113	—	150	7·4	—	5	—	78·5

The first of these features 100% PL activity, 26·5% β-glucosidase activity, a 34·5% reduction in melanoidins and a factor of purification from extraneous proteins of three. This preparation could be used on must and pressed grapes only when the advantage deriving from must fluidification is greater than probable disadvantages in the colour.[23] The second feature is characterized by 60% β-glucosidase activity and a 78·5% reduction in brown pigments, does not contain any PL and has a purification factor of five.

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