0273-2289/02/101/0001/$13.25

Inexpensive Isolation of β-D-Glucopyranosidase from α-L-Arabinofuranosidase, α-L-Rhamnopyranosidase, and *o*-Acetylesterase

Riccardo N. Barbagallo,* Giovanni Spagna, Cristina Abbate, Giuseppe Azzaro, and Rosa Palmeri

University of Catania, Biotechnology Group from the Department of Horticulture, Floriculture, Arboriculture and Agroindustrial Technology (DOFATA), Via S. Sofia 98, 95123 Catania, Italy, E-mail: guiding@tiscalinet.it and spggnn@iol.it

Received December 15, 2000; Revised March 22, 2001; Accepted October 4, 2001

Abstract

β-D-Glucopyranosidase (βG, EC 3.2.1.21) has been isolated from some collateral activities, α-L-arabinofuranosidase (Ara, EC 3.2.1.55), α-L-rhamnopyranosidase (Rha, EC 3.2.1.40), and *o*-acetylesterase (Est, EC 3.1.1.53), using a commercial enzyme preparation and a simple method economically sustainable for the food industry. The procedure comprises precipitation of extraneous substances by adding ethanol and $CaCl_2$, ultrafiltration, and adsorption, first on bentonite and then on chitosan. The results obtained were the complete isolation of βG from the above-mentioned activities, a drastic reduction in extraneous compounds, such as brown substances and polysaccharides, and a slight increase in purification.

Index Entries: β-D-Glucopyranosidase isolation; α-L-arabinofuranosidase; α-L-rhamnopyranosidase; *o*-acetylesterase; brown substances and polysaccharides.

Introduction

Many microorganisms produce a wide variety of enzymes (proteases, pectinases, cellulases, hemicellulases, and glycosidases) currently used in food technology *(1–4)*; in particular, among glycosidases, β-D-glucopyranosidase (βG, EC 3.2.1.21) is one of the most interesting, especially for enhancing the aroma of fruit juices, musts, wines, and other alcoholic beverages *(5–17)*. In many fruits and plant tissues, the chemical compounds that contribute to the aroma and fragrances occur in bond form and contain

*Author to whom all correspondence and reprint requests should be addressed.

β-D-glucopyranose linked directly to aglycon and/or indirectly to other sugars. These conjugate compounds, known as glycosides, are nonvolatile and are generally water soluble *(18)*. They can be perceived by the consumers following removal of the carbohydrate moiety, by acid or enzymatic hydrolysis, and consequent release of the volatile part (geraniol, nerol, citronellol, linalool, α-terpineol, etc.) *(19–21)*. Given its high specificity, enzymatic hydrolysis releases the terpenols bound to the glycosidic residues more rapidly and in a selective manner without bringing about structural alterations to the monoterpenes, thus developing a more natural aroma *(22)*.

The aim of this study was to develop a method for the complete isolation of βG from some collateral glycosidic activities, α-L-arabinofuranosidase (Ara, EC 3.2.1.55), α-L-rhamnopyranosidase (Rha, EC 3.2.1.40), and *o*-acetylesterase (Est, EC 3.1.1.53), using a commercial enzyme preparation and a simple method economically sustainable for the food industry. In our two previous works *(23,24)*, procedures were set up to increase the purification of βG tested along with Ara and Rha; however, in this study we examine the possibility of developing a method specifically aimed at eliminating the collateral activities that can affect the aromatic profile of the beverages and, at the same time, obtain a reduction in extraneous compounds, such as brown substances, polysaccharides, and intermediates of the Maillard reaction, and a slight increase in purification.

Materials and Methods

Materials

Three batches of the following enzyme preparations were used: AR 2000 (Gist Brocades, France), Novarom G (Novo Nordisk, Switzerland), Ultrazym 100 (Ciba-Geigy, Italy), Vinozym FCE G (Ciba-Geigy, Italy). The synthetic substrates used for activity assays were *p*-nitrophenyl β-D-glucopyranoside (*p*NPG), *p*-nitrophenyl α-L-arabinofuranoside (*p*NPA), *p*-nitrophenyl α-L-rhamnopyranoside (*p*NPR), and *p*-nitrophenyl acetate (*pNPAce*) (Sigma Chemical Co.). The adsorbents were Fractosil (Biorad, Italy), Silicagel 60 (Sigma), α-alumina (Sigma), hydroxyapatite (Sigma), bentonite (BDH, Switzerland), and chitosan (38–75 mesh, Protan, USA). Protein concentration was determined after precipitation in 7% (w/v) trichloroacetic acid by means of Coomassie Blue G250 (Serva, Germany) and employing bovine serum albumin as a standard (BSA, Sigma). All other reagents were of high purity and supplied by Carlo Erba (Italy), with the exception of the salts used for precipitation, $CaCl_2$ and KCl (Merck, Italy).

Enzymatic Assays

β-D-Glucopyranosidase (βG), α-L-rhamnopyranosidase (Rha), and α-L-arabinofuranosidase (Ara) activities were determined according to the method of Spagna et al. *(23)*, while *o*-acetylesterase (Est) activity was determined according a modified spectrophotometric method *(25)*. One gram of *p*-nitrophenyl acetate was added with 1 mL of acetone and, subsequently,

with a solution of the surfactant Tween 80 at 0.50% in 0.01 *M* citrate–citrate (C–C) buffer at pH 4. Fifty microliters of enzyme, dissolved in C–C buffer, were added by stirring at 32°C to 450 µL of the previously prepared solution. After 3 min the reaction was stopped by adding 2 mL of ethanol and the yellow coloring, developed by the released *p*-nitrophenolate ion, highlighted with the addition of 0.01 *M* C–C buffer at pH 6.0. The absorbance of the samples were read spectrophotometrically against the blank at λ of 333 nm.

The parameters considered in the purification were Recovery (Rec) = (final activity/initial activity) × 100; Purification (Pur) = (initial specific activity/final specific activity); DAbs = [(initial Abs – final Abs)/initial Abs] × 100, where Abs was the absorbance at 420 nm; DPol = (final Pol/initial Pol) × 100, where the initial Pol is the dry weight (at 75–80°C for 18–22 h) of precipitate of the enzymatic solution after diluting the solution with four volumes of ethanol and the final Pol which is eliminated by precipitation or adsorption. One unit of enzyme was defined as the amount of enzyme released by 1 µmol of *p*-nitrophenol min^{-1} under assay conditions.

Protein determination was carried out by colorimetric reaction with Coomassie Blue G250 according to Bradford *(26)*.

Isolation Methodology

Precipitation Trials

Ethanol: A sample of 8.6 g of Novarom G was dissolved in 50 mL of 0.01 *M* C–C buffer at pH 4.0. Absolute ethanol at a low temperature (3–4°C) was added very slowly to the enzymatic solution kept under stirring in order to obtain final solutions between 10% and 50% (v/v). The samples were left at 3–4°C for 2.5 h, after which the supernatants were separated by centrifugation at 4100*g* at 3°C for 20 min and immediately analyzed.

Salts: Six solutions were prepared from a solution of the commercial enzyme of 8.6 g in 50 mL of 0.01 *M* buffer C–C at pH 4.0. The chosen salts (KCl, $CaCl_2$) were slowly added at increasing concentrations (respectively, 0.2, 0.5, 1 *M* for KCl, and 0.1, 0.2, 0.5 *M* for $CaCl_2$). The maximum level of concentration matched maximum solubility in 0.01 *M* C–C buffer solution at pH 4.0 and 30% ethanol. The suspensions obtained tended to form a gel, but were immediately centrifuged at 4100*g* for 30 min and the supernatants analyzed thereafter.

Adsorption Trials

Suspensions of various adsorbents (Fractosil, Silicagel, α-alumina, hydroxyapatite, bentonite, and chitosan) were prepared at a concentration of 2% (w/v) in 0.01 *M* C–C buffer at pH 5.6. The bentonite was dispersed for about 30 minutes by means of a small spatula. Two milliliters of such suspension were added to 15 mL of enzyme preparation that had previously undergone precipitation with ethanol and 0.5 *M* $CaCl_2$, filtration with Whatman paper 42 (diameter 90 mm), and ultrafiltration. The system employed was produced by Millipore and used a membrane Pellicon XL with a cut-off of 50,000 kDa. A further 20 mL aliquot of suspension was

added directly to 15 mL of Novarom G dissolved in the same buffer following treatment with 30% ethanol. The tubes were stirred for around 2.5 h at 25°C, then centrifuged at 4100*g* for 20 min at 25°C.

Final Process

A sample of 86.0 g of Novarom G was dissolved by stirring in 350 mL of 0.01 *M* C–C buffer at pH 4.0. Next, 150 mL of cold ethanol (30% v/v) were added slowly to the solution. The solution thus formed was left to stand for 2.5 h at 3–4°C, following which the precipitate formed was removed by centrifugation at 4100*g* for 20 min at 3°C. The supernatant containing the glycosidases was treated immediately with 0.5 *M* $CaCl_2$, then centrifuged under the same conditions, filtered with Whatman paper 42 (diameter 90 mm), and ultrafiltered using a membrane Pellicon XL with a cut-off of 50,000 kDa. The enzyme was quantitatively recovered from the membrane with 0.01 *M* buffer C–C at pH 5.6 and made up to a total volume of 260 mL. The solution thus obtained was placed in contact with bentonite and subsequently with chitosan at a concentration of 2% (w/v) for around 3 h at a temperature of 25°C. The surnatant was recovered after centrifugation at 4100*g* for 20 min at 25°C.

Chemical and Physical Enzyme Characterization

The following parameters were determined for βG: optimum pH (between 3.0 and 7.0 in 0.1 *M* C–C buffer) at 30°C; optimum temperature (between 20 and 90°C) at pH 4.0; K_m and V_{max} (at 30°C and pH 4.0).

For the SDS-PAGE, 3 µg of protein samples were reduced with 100 m*M* DDT and boiled for 5 min prior to analysis. Samples were analyzed on pre-cast 10% Tricine gels using a Novex Xcell II mini system (Novex, San Diego, CA) and Tricine SDS running buffer according to the manufacturer's instruction. High-molecular-weight markers (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK) ranging from 14,500 to 97,000 kDa were analyzed concurrently.

Results and Discussion

Selection of the Enzyme Preparation

In making this selection, various parameters were considered: enzyme activity, proteins, specific activity, ratio between βG and other enzyme activities, as well as the ratio between activity and absorbance at 420 nm, which is considered an indicator of the presence of brown compounds resulting from Maillard's reaction during sterilization and/or fermentation of enzyme production from broth. Adding brown compounds to beverages can lead to a change in their sensory characteristics; in addition, if the enzymes need to be used for immobilization, the brown compounds generally have a negative influence, owing to being adsorbed aspecifically by the various supports in such a way as to compete with the enzyme in bonding itself to the reactive centers of the matrix. Table 1 details the character-

Table 1
Analysis of βG, Ara, Rha, and Est Contained in Various Commercial Products[a]

Products	Enzyme	Activity (U g^{-1})	Proteins (mg g^{-1})	Specific activity (U mg^{-1} proteins)	βG/Ara	βG/Rha	βG/Est	βG/(Ara+ Rha + Est)	Activity/ Abs
AR 2000	βG	158	31.60	5.00				0.30	1.88
	Ara	503		15.91	0.31				
	Rha	8.16		0.25		19.4			
	Est	22.9		0.72			6.9		
Novarom G	βG	201	41.47	4.85				11.67	4.37
	Ara	14.0		0.33	12.01				
	Rha	1.0		0.02		157			
	Est	2.23		0.05			90.1		
Ultrazym	βG	5.92	8.21	0.72				1.38	1.21
	Ara	2.82		0.34	2.1				
	Rha	0.50		0.06		11.8			
	Est	0.98		0.12			6.04		
Vinozym	βG	0.21	5.72	0.04				0.0029	0.18
	Ara	66.90		11.70	0.0031				
	Rha	2.83		0.49		0.0742			
	Est	2.28		0.40			0.0921		

[a]The enzyme assay was made at 32°C and pH 4.0. Three batches of each commercial preparation were analyzed. The variability in activity of the glycosidases among the batches was ± 8%.

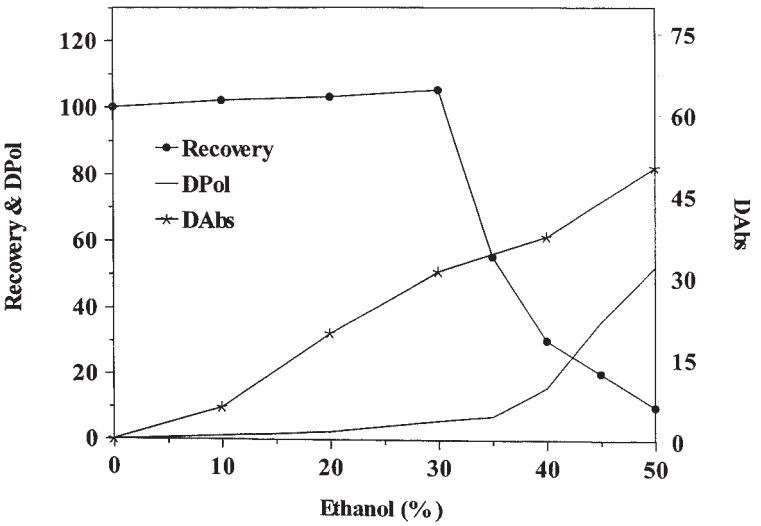

Fig. 1. Recovery values of βG, DPol, and DAbs as a function of ethanol concentration.

istics of the assayed enzyme preparations, all used by the European food industry and obtained from *Aspergillus niger*.

Novarom G was chosen from among the various commercial preparations for the highest βG activity values and more favorable ratio with all collateral glycosidic activities. Subsequently, three batches of Novarom G were mixed for the isolation trials.

Precipitation Trials

The principle adopted for this enzyme purification consists in the precipitation of extraneous substances (particularly proteins and polysaccharides) by adding organic solvents or salts (salting-out).

Ethanol was chosen because βG is a fairly stable enzyme in the presence of this organic solvent *(23)* and the commercial enzymes containing it are used in alcoholic beverages. The addition of ethanol to the enzyme solution promotes solute–solute interactions (especially among the polar macromolecules with a large surface area and molecular weight) by lowering the solution's dielectric constant and polarity. Nevertheless, ethanol can deactivate the enzymes, by unfolding their native conformation, and cause their precipitation *(27)*, by increasing the intermolecular interactions. In order to reduce such an effect, cold ethanol was used for a short time. This step was followed by centrifugation to eliminate the gelatinous precipitate.

Novarom G was tested at increasing concentrations of ethanol (from 10% to 50%) in order to determine the optimum concentration at which there is maximum elimination of polysaccharides and minimum loss in βG activity. Figure 1 shows that 30% ethanol concentration represents the best compromise between recovery of βG and reduction in polysaccharides

(DPol) and brown compounds (DAbs). In fact, the trials show that at this concentration of ethanol there is a 105% recovery of βG, 5.7% of DPol and 31.2% of DAbs.

In order to achieve greater recovery of βG compared to the other enzymes, precipitation tests with salts (KCl, $CaCl_2$) were carried out. By salting-out, the salt ions coordinate the solvation shell of the macromolecules that thus interact among themselves and precipitate selectively mainly on the basis of polarity and molecular weight/volume ratio.

It is worth noting that by adding the salts ($CaCl_2$ and KCl) together to the ethanol, without a preliminary centrifugation, considerable precipitation was noted but βG recovery was nevertheless low (<12%). This could be due to the inclusion of the enzyme in the gelatinous precipitate. On the other hand, adding the salts after having eliminated the precipitate from the ethanol by centrifugation allowed much higher recovery of βG (Table 2). The use of 0.5 *M* $CaCl_2$ was preferred because it allowed the greatest fall in Rha with a high βG/Rha ratio and high purification and recovery values.

At this point ultrafiltration was effected. The operation was preceded by filtration in order to eliminate the slow but irreversible formation of a precipitate on the surnatant that was probably composed of calcium pectate.

As can be noted from Table 3, in this step the Est was eliminated. The Rha was reduced, probably as a result of the lower molecular weight of this enzyme (MW 90,000 kDa) compared to the βG (MW 120,000 kDa) *(28)*.

Adsorption Trials

After ultrafiltration the following adsorbents were tested: α-alumina, bentonite, chitosan, Fractosil, hydroxyapatite, Silicagel, under the same conditions adopted in a previous study *(23)*. The same supports were also tested prior to ultrafiltration, however, the results obtained were very low. In particular, bentonite produced no fall in collateral activities due to the presence of calcium ions in solution that probably saturated the supports' negative adsorption sites, thus preventing the "capture" of foreign proteins. This inevitably means that ultrafiltration has to be carried out prior to adding the various adsorbents.

Table 3 shows that unlike the other supports used, treatment with bentonite provides a notable increase in βG/Rha. The adsorption of the Rha on the bentonite could involve different types of interactions. At adsorption pH, Rha is positively charged (p*I* 6.0) *(29,30)* so that it can be adsorbed by electrostatic interaction on the negative laminar layers of the bentonite. This charge is due to the replacement of Si^{+4} with other metals having a lower valence such as Al^{+3} with consequent negative-ion vacancies. Other probable interactions include the formation of coordination bonds of the Rha with the cations present on the surface of the support *(31)*.

It should be pointed out that a test was also carried out with bentonite at a concentration of 2.0% directly on the enzyme preparation treated at 30% ethanol, following correction of pH to 5.6. In this case, in addition to

Table 2
Precipitation of Extraneous Substances Through the Addition of Ethanol and Salts (Salting-Out)

Products	Concentration (*M*)	Activity (U g^{-1})				Proteins (mg g^{-1})	Specific activity (U mg^{-1} proteins)	βG/ Ara	βG/ Rha	βG/ Est	βG/ (Ara + Rha + Est)	βG Rec (%)	βG Pur
		βG	Ara	Rha	Est								
Ethanol 30%	–	211	12.65	0.72	1.52	45.74	4.61	16.67	293	138.9	14.4	100	1.0
$CaCl_2$	0.1	209	12.44	0.65	1.50	44.35	4.71	16.80	321.5	139.3	14.32	99	1.0
	0.2	205	12.19	0.59	1.44	43.40	4.72	16.82	347.4	142.4	14.42	97	1.0
	0.5	204	11.76	0.26	1.40	31.84	6.41	17.35	784.6	145.7	15.20	97	1.4
KCl	0.1	202	12.61	0.59	1.50	43.30	4.65	16.01	342.4	134.7	13.70	95	1.0
	0.5	196	12.45	0.46	1.30	36.88	5.31	15.74	426.1	150.8	13.79	93	1.2
	1.0	161	11.92	0.40	1.20	31.82	5.06	11.91	402.5	134.1	12.00	76	1.0

Table 3
Adsorption of Extraneous Substances on Various Supports[a]

Adsorbents	Activity (U g^{-1})				Proteins (mg g^{-1})	Specific activity (U mg^{-1} proteins)	βG/Ara	βG/Rha	βG/(Ara+ Rha)	βG Rec (%)	βG Pur
	βG	Ara	Rha	Est							
UF	202	9.38	0.14	0	30.0	6.73	21.53	1443	21.22	101	1.4
α-Alumina	162	8.91	0.13	0	28.2	5.74	18.1	1246	17.92	80	0.85
Bentonite	160	8.76	0.02	0	23.7	6.75	18.26	8000	18.22	79	1.0
Chitosan	157	3.59	0.12	0	27.3	5.75	43.7	1304	42.32	78	0.85
Fractosil	163	8.50	0.14	0	26.8	6.08	19.1	1164	18.87	81	0.90
Hydroxyapatite	164	8.30	0.11	0	29.0	5.66	19.7	1491	19.50	81	0.84
Silicagel	169	8.0	0.14	0	28.4	5.95	21.1	1207	20.80	84	0.88

[a]The tests were conducted at a concentration of 2%, temperature 25°C, and pH 5.6.

the fall in Rha, Ara, and Est, there was at the same time an excessive fall in βG activity amounting to 42%.

Ara is considerably reduced following "capture" by the chitosan; the positively charged chitosan essentially behaves as a polycation and so can adsorb this negatively charged enzyme (p*I* 3.5) by electrostatic interaction thanks to its protonable amino groups *(32)*.

Such an effect is smaller for βG because this enzyme has an p*I* of 4.2 and thus narrow the range in which it can be adsorbed by the chitosan. At concentrations higher than 2%, recovery and purification decreased; this could be attributed to steric inclusion phenomena, given the ability of chitosan to swell and gel in an acid environment. Moreover, brown compounds, proteins, and polysaccharides could partially reduce the electrostatic repulsion and mediate the adsorption of βG on chitosan.

At this point, the sample was subjected to ultrafiltration with bentonite followed by chitosan or vice versa, but the best results were obtained by the sequence bentonite–chitosan that allows complete isolation of βG from residual Ara and Rha. Both adsorbents are easy to get hold of and very cheap.

Final Process

The final purification scheme proposed (Table 4) combines several of the previous steps that offered the best results.

The step with ethanol provides a reduction of impurities (DAbs and DPol) and good recovery. The step with $CaCl_2$ allows the activity of Rha to be minimized. The ultrafiltration step, necessary in order to eliminate the ethanol that would otherwise have inactivated the βG, also removes the Est and further reduces the amount of the brown compounds and polysaccharides. The treatment with adsorbents on the prepurified enzyme shows better results compared to the raw one. This is probably due to the fact that it acts on a "cleaner" system, particularly as regards brown compounds that as previously noted demonstrate high affinity for such an adsorbent. Moreover, a large reduction in brown compounds can be noted. In particular, the treatment with bentonite results in the elimination of Rha, while final adsorption with chitosan allows complete isolation of βG from Ara, with a slight increase in purification, albeit with a reduction in enzyme recovery.

Enzyme Characterization

The optimum pH value for βG was found to be 4.2 with a relative activity of about 75% for the enzyme at pH 4.0. The optimum temperature was found to be about 65°C for βG. Finally, the V_{max} value was 2.2 U mg^{-1} protein, while the K_m value was 9.1 m*M*.

Figure 2 shows the SDS-PAGE of the steps of the final isolation process. As can be observed, following ultrafiltration, there is a reduction in proteins with MW of less than 66,000 kDa, as well as after the absorption on

Table 4
Final Isolation Process

Step	Enzyme	Activity (total units)	Protein (total mg)	Specific activity (U mg^{-1} proteins)	βG/Ara	βG/Rha	βG/Est	DAbs (%)	DPol (%)	βG Rec (%)	βG Pur
Novarom G	βG	17,286	3,566	4.85				0	0	100	1.0
	Ara	1,204		0.34	14.36						
	Rha	86.00		0.02		201					
	Est	191.78		0.53			90.13				
Ethanol	βG	18,146	3,934	4.59				31.2	5.70	105	1.0
	Ara	1,088		0.28	16.68						
	Rha	61.92		0.02		293.06					
	Est	130.72		0.03			138.82				
$CaCl_2$	βG	17,544	2,738	6.41				45.61	8.16	101	1.3
	Ara	1,011		0.37	17.35						
	Rha	22.36		0.0082		784.62					
	Est	120.4		0.0044			145.71				
UF	βG	17,372	2,580	6.73				58.0	16.70	100	1.4
	Ara	806.68		0.31	21.54						
	Rha	12.04		0.0046		1442.86					
	Est	0					–				
Bentonite	βG	13,760	2,038	6.75				74.0	70.10	80	1.4
	Ara	753.36		0.37	18.26						
	Rha	0		–		–					
	Est	0		–			–				
Chitosan	βG	10,750	1,118	9.62				88.0	78.4	62	2.0
	Ara	0		–	–						
	Rha	0		–		–					
	Est	0		–			–				

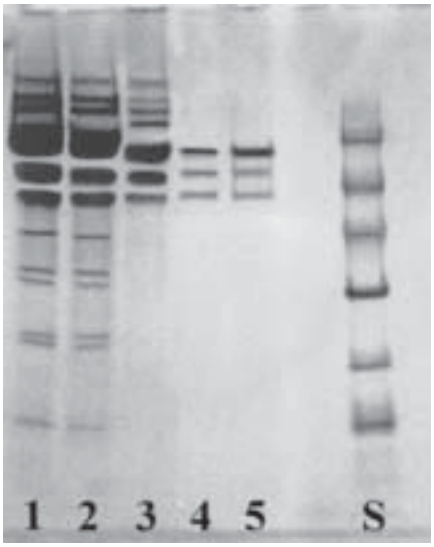

Fig. 2. SDS-PAGE of the various steps of βG isolation. From left to right: lane 1, crude enzyme; lane 2, after ethanol precipitation; lane 3, after ultrafiltration; lanes 4–5, after adsorption with bentonite and chitosan, respectively; lane S, marker proteins (Amersham Pharmacia Biotech, ranging from 14,500 to 97,000 kDa).

bentonite for the proteins with MW exceeding 97,000 kDa. On the basis of the characteristics of the βG, the 95,000 kDa lane ought to be that of the enzyme.

Conclusions

In our previous studies *(23,24)* we developed procedures aimed at increasing the purification of some glycosidases. In the present study for the first time has been possible to obtain the complete isolation of βG from collateral glycosidic activities and *o*-acetylesterase contained in a commercial preparation currently utilized in the food industry. This result alone represents the major strongpoint of the method adopted that also provides a slight increase in purification, with a satisfactory reduction in brown compounds and polysaccharides. The method has proved simple and economically viable for application in the food industry.

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