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Purification of Yeast Periplasmic Proteins Using Protein Adsorption to Glucan

*Renata Teparić and Vladimir Mrša**

Laboratory of Biochemistry, Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, 10000 Zagreb, Croatia.

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Summary

Partially purified yeast glucan was used for the one-step purification of acid phosphatase from the Saccharomyces cerevisiae cell extract, as well as for the purification of invertase from several commercially available preparations. In both cases pure enzymes, as judged by electrophoresis, were obtained by the adsorption of impurities to glucan. The method described is simple, provides high yields, causes no significant protein denaturation and is inexpensive. It can most probably be used for other yeast extracellular proteins, as well as for proteins from other sources which have low binding potential for yeast glucan.

Keywords: acid phosphatase; invertase; protein adsorption to glucan

Introduction

Studies of the molecular organization of the *Saccharomyces cerevisiae* cell wall have revealed that glucan, the major structural carbohydrate component of the wall, efficiently binds proteins *in vitro (1)*. The binding was found to be due to relatively unspecific hydrogen-bonding of the carbohydrate moiety with a number of yeast proteins, as well as with many proteins from other sources. It was also found that the binding was strictly pH-dependent, so that proteins adsorbed to glucan only at pH lower than 7.0 *(1)*. Similar interactions have also been observed with yeast chitin, although the capacity of this polysaccharide for the binding of proteins was lower than that of glucan *(2,3)*. The third yeast wall carbohydrate, mannan, as well as cellulose, were inactive and showed no interactions with proteins at any pH.

Protein-carbohydrate interactions were found to be unspecific so that many proteins adsorbed to glucan. However, exceptions were found among yeast extracellular proteins: periplasmic proteins invertase and acid phosphatase *(1)*, and exo-β-glucanase (Exg1p) *(3)*, which was reported to be secreted into the yeast growth medium *(4,5)*. The first two proteins failed to react with glucan under the conditions required for the binding of other proteins, while the glucanase interacted with the polysaccharide with a much lower affinity *(3)*. Since most yeast extracellular proteins, including invertase and acid phosphatase, are glycosylated, it was investigated whether the carbohydrate moiety of these glycoproteins prevented their contact with glucan. Results showed, however, that even the nonglycosylated intracellularly located invertase failed to interact with glucan, suggesting that it was the property of the protein part of the molecule.

The fact that yeast extracellular proteins, unlike most other proteins, do not adsorb to glucan led to the idea to use this property for their purification. Indeed, a simple method for obtaining highly purified acid phosphatase from *Saccharomyces cerevisiae* cells grown in a low-phosphate medium, as well as for the purification of invertase from commercially available preparations to homogeneity, is presented in this paper. Since invertase, as well as acid and alkaline phosphatases from different sources, represent enzymes with a number of industrial and laboratory applications, the method described could have a particular significance from the biotechnological point of view.

Materials and Methods

Yeast strain, medium and growth conditions

Saccharomyces cerevisiae strain P-28-24-C (a, pho3-1, PHO5) was used in this work. Cells were grown in the low phosphate YPD medium for the derepression of acid phosphatase as described previously *(6)*.

* author to whom correspondence should be addressed

Materials

Saccharomyces cerevisiae invertase was obtained from Boehringer (Manheim, Germany), or Fluka (Buchs, Switzerland).

Yeast glucan was prepared by a modification of the method of Peat *et al.(7)*. Fresh commercial yeast (160 g) was suspended in 128 mL of 6% NaOH and heated for 2 hours at 60 °C. After that, the mixture was neutralized by the addition of 128 mL 1.5 M HCl and centrifuged 10 minutes at 12000 rpm. Glucan precipitate was washed 3 times with water and then with 0.1 M Na-acetate buffer pH = 3.8 until the constant pH was achieved. This procedure contains only the first step of the glucan purification described *(7)*, and the material obtained still contains some impurities. However, no soluble, or protein impurities were detected, and further purification was not required for the procedure for the purification of periplasmic proteins described here.

All other chemicals used in this work were of the highest purity grade available.

Purification of acid phosphatase

Yeast cells grown under derepressing conditions (100 mL of low phosphate medium, $A_{600} = 4$) were harvested by centrifugation and suspended in 1.6 mL of 0.05 M Na-acetate buffer pH = 3.8. The same volume of glass beads (0.5 mm in diameter) was added and cells were disrupted by shaking the suspension for 2 minutes (4000 rpm) in the Braun MSK homogenizer. Cell debris was centrifuged 20 minutes at 12000 rpm after which 45 mg of glucan and the same amount of glass beads as for the cell disruption was added to the supernatant and the mixture was shaken in the Braun homogenizer for additional 2 minutes. This step allows efficient adsorption of proteins to glucan. The suspension was centrifuged 10 minutes at 3000 rpm, and the supernatant was analyzed by electrophoresis.

Purification of invertase

Commercial preparation of invertase was dissolved in 10 mM Na-acetate buffer pH = 4.5 (20 mg/mL), and dialysed overnight in the same buffer. Glucan (27.5 mg) was suspended in 0.15 mL of the invertase solution. The same volume of glass beads was added and the mixture was vortexed 10 minutes at room temperature. The suspension was centrifuged 8 minutes in an Eppendorf centrifuge and the supernatant was analyzed by electrophoresis.

Electrophoresis

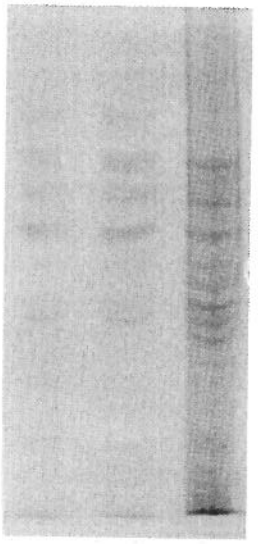
Electrophoresis was performed essentially by the method of Laemmli *(8)* on gel slabs consisting of a 10% separating gel and a 4.5% stacking gel. Proteins were stained by immersing the slabs in 0.2% Coommassie Brilliant Blue R-250 in 40% methanol, 10% acetic acid, and subsequently destained in 7% acetic acid. Samples applied contained 10–20 μg of proteins.

Other methods

Proteins were determined by the Lowry method *(9)*. Acid phosphatase activity was determined using p-nitrophenyl phosphate as substrate *(10)*. Invertase activity was assayed as described *(11)*.

Results and Discussion

The observation that yeast glucan binds proteins nonspecifically by adsorbing them at acidic pH, pointed out the potential significance of pH during the mechanical disruption of cells in order to obtain intracellular proteins. If the disruption is carried out at acidic pH, lower yields could be expected due to the retention of liberated intracellular proteins by the glucan containing cell debris. To check if this is so, *Saccharomyces cerevisiae* cells were grown to the early logarithmic phase and disrupted in the Braun homogenizer with glass beads at different pH. Solubilized proteins were quantified and analyzed electrophoretically. Results are presented in Fig.1. and it can be seen that the release of proteins at basic pH was indeed much better than that in the acidic pH region. The yield of total intracellular proteins was 3-fold higher when cells were broken at pH = 8.0, than at pH = 4.5. Such results showed that it would be desirable to suspend yeast cells in neutral or basic buffers for the mechanical disruption if experimental requirements allow it. They could also explain the relative simplicity of the purification of yeast periplasmic proteins, particularly acid phosphatase *(12,13)*, which is active and stable in the acidic pH region. Therefore, isolation and purification procedures, including the cell disruption, were normally performed at pH about 4.0, eliminating in this way a large amount of intracellular proteins already during the cell breakage by adsorbing them to cell debris, thus giving unusually high starting specific activi-



pH	*m*(proteins) / mg*
4.5	0.040
6.0	0.048
8.0	0.120

* mass obtained from 1 mL of medium $A_{600} = 4$

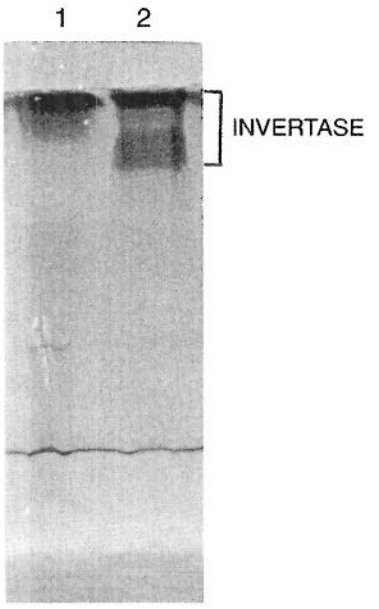
Fig. 1. Intracellular proteins released by cell disruption at different pH. Lanes: 1. pH = 4.5; 2. pH = 6.0; 3. pH = 8.0. Amounts of released proteins are given in the table.

	1	2
Activity / μmol min^{-1}	7.30	7.50
m (proteins) / mg	1.95	1.56

Fig. 2. Purification of acid phosphatase by adsorption of impurities to glucan. Lanes: 1. crude extract; 2. preparation after the reaction with glucan. Amounts of proteins and phosphatase activity are given in the table.

ties of acid phosphatase. However, extracts still contained a significant amount of impurities, still requiring ammonium sulphate precipitation and several chromatography steps for the complete purification of the enzyme *(12)*. Therefore, the repeated adsorption of protein impurities to glucan was attempted in order to simplify the purification of the enzyme. Results of the procedure described in the Materials and Methods section are shown in Fig. 2. It can be seen that already one glucan-binding step was sufficient to obtain acid phosphatase practically purified to homogeneity, thus replacing several purification steps described previously *(12)*. Vigorous shaking of the mixture was required to allow contact and interactions between proteins and glucan, so that the best results were achieved if the procedure was done in the homogenizer under the same conditions as cell disruption. No significant loss of enzyme activity was recorded, but it should be kept in mind that the application of the method for other extracellular proteins might require milder, and accordingly longer, vortexing conditions. It has to be mentioned that a relatively high amount of proteins was applied to electrophoresis shown in Fig. 2 (about 20 μg per lane). The acid phosphatase band still appears relatively weak due to the heterogeneity of the enzyme, resulting in a difuse band, and poor binding of the dye, because of the extensive glycosylation. However, the gel confirms the removal of practically all visible impurities.

To investigate whether the method worked out with acid phosphatase could be applied to other yeast extracellular proteins, the purification of *S. cerevisiae* invertase was studied. Invertase is also stable in acidic buffers, but the region of stability is somewhat higher than that for acid phosphatase *(14)*. Therefore, the glucan adsorption step was done at pH = 4.5. Since a higher susceptibility of the enzyme to mechanical shaking was observed, the reaction was performed by vortexing in Eppendorf tubes as described under Materials and Methods. When the method was used for the purification of invertase from crude extracts of yeasts grown under derepression (low glucose) conditions , it was not possible to achieve the complete purification, although after two adsorption steps the specific activity of the enzyme increased 3–4 times (not shown). This procedure can therefore be used only as one of the steps in the invertase purification, but additional chromatographies are still required. Invertase can also be purchased commercially, but electrophoresis of commercial preparations reveals that they still contain protein impurities. The glucan adsorption method was used successfully to remove these impurities as can be seen in Fig.3, yielding an apparently homogenous invertase preparation. The shift of the invertase band towards smaller molecular masses is most probably due to the change in the oligomeric structure of the protein *(15,16)*, but it does not cause a change in the enzyme activity. A



	1	2
Activity / μmol min^{-1}	4600	3145
m (proteins) / mg	2.37	2.19

Fig. 3. Purification of commercial invertase preparation (Fluka) by adsorption of impurities to glucan. Lanes: 1. commercial preparation; 2. purified invertase. Amounts of proteins and invertase activity are given in the table.

somewhat lower specific activity of purified invertase is, rather, a consequence of the relative instability of the enzyme under the purification conditions.

In conclusion, this paper presents a simple method for the purification of yeast periplasmic proteins acid phosphatase and invertase, enzymes with a potential technological significance. Additional experiments are required to investigate the possibility that extracellular proteins from other cells which contain glucan in their cell walls (assuming that they do not bind to glucan) could be purified using the same procedure. The described method is quick, efficient and inexpensive, and requires no unusual chemicals or equipment. Therefore, it could be used as a valuable tool for simple and quick purification of extracellular proteins to homogeneity, or, in some cases, could represent one of the steps in their purification procedure.

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Pročišćavanje periplazmatskih proteina kvasca s pomoću adsorpcije proteina na glukan

Sažetak

Djelomično pročišćeni glukan kvasca upotrijebljen je za pročišćavanje kisele fosfataze iz staničnog ekstrakta kvasca ***Saccharomyces cerevisiae*** *u jednom koraku, kao i za pročišćavanje invertaze iz nekoliko pripravaka dostupnih na tržištu. U oba slučaja adsorpcijom onečišćenja na glukan dobiveni su čisti enzimi, kako je potvrđeno elektroforezom. Opisana metoda je jednostavna, omogućuje dobro iskorištenje, ne uzrokuje znatniju denaturaciju proteina i nije skupa. Vjerojatno se može primijeniti i za druge kvaščeve izvanstanične proteine, a i za proteine iz drugih izvora koji imaju mali potencijal za vezanje na glukan kvasca.*