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Chitosan as a macroaffinity ligand Purification of chitinases by affinity precipitation and aqueous two-phase extractions

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Abstract

(1) Chitosan was found to be a suitable macroaffinity ligand for affinity precipitation of chitinase from *Neurospora crassa*, cabbage and puffballs. (2) The activity recoveries of 85, 82 and 90% with concomitant fold purifications in terms of specific activities were 27, 15 and 30 with *N. crassa*, cabbage and puffballs and were obtained with affinity precipitation. These results were obtained with clarified extracts/homogenates as the starting materials. (3) The incorporation of chitosan in poly(ethylene glycol) (PEG)–salt aqueous two-phase system allowed purification of chitinases from these sources directly from unclarified extracts/homogenates. (4) The 96% (w/v) chitosan (of initially introduced into the aqueous two-phase system) partitioned into PEG-phase and this enhanced the partitioning of chitinases into PEG-phase. The chitosan, free as well as bound to chitinases, could be separated from PEG-phase by increasing the pH to 7. (5) By the process of desorption with 2.0 M $MgCl_2$, 86, 80 and 88% activity recoveries (% expressed in terms of total units of enzyme activities in the crude extract) were obtained in the case of *N. crassa*, cabbage and puffballs, respectively. The corresponding fold purifications in terms of specific activities were 34, 20 and 38. (6) The purified preparations gave single bands on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and the estimated molecular masses agreed with the reported values in the literature.

Keywords: Chitinases; Chitosan; Affinity precipitation; Two-phase affinity extraction; Protein purification; Polymers

1. Introduction

Chitin [(1 → 4)-2-acetamido-2-deoxy-β-D-glucan] occurs naturally in all crustaceans and is generated as a waste during processing of shellfish, crabs, shrimps and krill [1–3]. Chitosan is obtained by partial deacetylation of chitin. Chitin oligosaccharides have medical applications such as reduction of cholesterol concentration in plasma, accelerating wound healing and inhibition of bacterial growth [4]. Thus, chitinases are useful for obtaining value added products from a waste, which otherwise poses a disposal problem [5–6]. Chitinases have been purified by different protocols [7–9]. Often such purifications have been carried out by following multistep protocols [10,11]. Chitin has been reported to be an affinity matrix in some of these purification strategies [4,12]. The present work has used deacetylated chitin as an affinity ligand and to develop two protocols for purification of chitinases.

Two-phase aqueous extraction has been widely recognized as an efficient and scalable technique, which can directly deal with unclarified extracts/homogenates [13]. Kamihira et al. [14] and Guoquiang et al. [15] have reported purification of lactate dehydrogenase and recombinant protein A, respectively, by aqueous two-phase extraction integrated with affinity precipitation. Recently, we have described purification of amylases [16], xylanases [17] and phospholipase D [18] as

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further applications of this approach. In this integrated approach, a macroaffinity ligand was partitioned into top-phase. This helped in selective partition of the target protein in this phase. After this, the approach follows affinity precipitation protocol. Thus, in order to use this integrated approach, the first step is to check the viability of affinity precipitation for the specific application. In recent years, affinity precipitation has been successfully used as a purification technique for amylases [19–21], phospholipase D [22] and pullulanase [23]. In this technique, a smart macroaffinity ligand selectively precipitates the target protein in response to a suitable stimulus. Earlier, it has been shown that chitosan can be used as a macroaffinity ligand for some lectins [24] and lysozyme [25]. In this work, initially chitinases from *Neurospora crassa*, cabbage and puffballs have been purified by affinity precipitation using chitosan as a macroaffinity ligand.

Encouraged with the successful application of chitosan as a macroaffinity ligand for chitinase in affinity precipitation, the purification of the enzymes from unclarified extracts/homogenates has also been carried out by aqueous two-phase affinity extraction. This has been carried out by incorporation of chitosan in a poly(ethylene glycol) (PEG)–salt system.

Finally, the results from these two techniques (affinity precipitation as such and its integrated format with two-phase aqueous extraction) have been compared. Such comparisons are valuable since these provide some help to other workers to choose between various options.

2. Materials and methods

2.1. Materials

Chitosan (Cat. No. C-0792), chitin (from crab shells, Cat. No. C-7170) and *N*-acetyl-D-glucosamine were purchased from Sigma (St. Louis, MO, USA). Poly(ethylene glycol) 6000 was obtained from E. Merck, Mumbai, India. *N. crassa* culture was obtained from Indian Type Culture Collection (ITCC), Mycology Division, Indian Agricultural Research Institute (IARI), New Delhi, India. Puffballs were purchased from Snow view Mushroom Laboratory and Training Centre, New Delhi, India. Cabbage was obtained from local market. All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Culture conditions of *N. crassa*

N. crassa was conveniently grown at 25–28 °C in 5-gal polycarbonate carboys containing 15 L of minimal medium plus 2% sucrose [26]. Carboys were inoculated with 2×10^6 conidia (6 days old) per mL. The contents of the carboys were agitated for 48 h by air, forced through a sterile cotton filter. The mycelia were collected on a Buchner funnel covered with cheesecloth. The mycelial mass was washed thrice with distilled water and finally with buffer, and pressed against filter paper and the wet weight was noted. The mycelial mass was processed further for enzyme activity.

2.2.2. Preparation of enzyme extract from *N. crassa*

Procedure followed was of Bridge [27] with slight modifications. A 10% suspension of the mycelial mass was made in buffer and frozen overnight at −20 °C. Acid washed sand, four times the weight of the mycelium, was added, and the mixture was ground in a chilled pestle-motar kept in an ice bath. The homogenate was centrifuged at 12,000 × *g* for 30 min at 4 °C. The supernatant of mycelial extract was used for chitinase assay.

2.2.3. Preparation of enzyme extract from cabbage

Fresh cabbage (*Brassica oleracea*) leaves (250 g) were homogenized with 750 mL of 0.1 M acetate buffer, pH 5.2 in a mixer [28]. After the mixture was strained through four layers of cheesecloth, the turbid filtrate was centrifuged at 10,000 × *g* for 10 min (at 10 °C). The supernatant obtained was dialysed against several changes of 0.1 M acetate buffer, pH 5.2 and used as the chitinase extract.

2.2.4. Preparation of enzyme extract from puffballs

Puffballs (100 g) were washed and homogenized with 200 mL of 0.1 M acetate buffer of pH 5.0 for 1 h at 4 °C in a mixer. This homogenate was centrifuged at 10,000 × *g* for 20 min at 10 °C and used for chitinase enzyme [29].

Affinity precipitation, pH and temperature optimization work were carried out with clarified crude extract after centrifugation. The aqueous two-phase purification was carried out directly with an unclarified crude homogenate.

2.2.5. Preparation of chitosan solution

Chitosan is a cationic polysaccharide [30]. It becomes reversibly soluble (pH < 7) and insoluble (pH > 7). Chitosan solution was prepared by dissolving 1 g of chitosan in 30 mL of 1% acetic acid and then precipitation was carried out by raising the pH to 8.0 by addition of 3 M NaOH. The precipitated chitosan was washed three times with 0.01 M Tris–HCl buffer, pH 8.0 and again solubilized in 50 mL of acetate buffer, pH 5.0 to get the final concentration of 2% (w/v).

2.2.6. Preparation of chitin suspension

The chitin suspension was prepared by adding 1 g of chitin in 100 mL of buffer (0.1 M Tris–HCl, pH 6.5 for *N. crassa*; 0.1 M acetate buffer, pH 5.2 for cabbage and 0.1 M acetate buffer, pH 5.0 for puffballs, respectively) and stirring it for 3 h at 25 °C. The suspension was stirred again before use.

2.2.7. Determination of enzyme activity

Chitinase activity using chitin, as substrate, was determined, as described by Chang et al. [28]. One unit of enzyme activity is defined as the amount of enzyme required for the formation of 1 μmol of the product (estimated as *N*-acetylglucosamine) per minute, under standard assay

conditions. The amount of reducing sugar was estimated by dinitrosalicyclic acid method [31]. The activity measurements were carried out by withdrawing aliquots in the range of 100–500 μL.

2.2.8. Protein estimation

Protein concentration was estimated by the dye-binding method using bovine serum albumin as standard [32]. These estimations were done by withdrawing aliquots in the range of 100–500 μL.

2.2.9. Purification of chitinase by affinity precipitation

The chitinase extracts from *N. crassa*, cabbage and puffballs (1.0 mL containing 15, 27 and 35 U, respectively) were added separately to 0.5% (w/v) of chitosan solution and 2.75 mL of the buffer (0.1 M Tris–HCl, pH 6.5 for *N. crassa*; 0.1 M acetate buffer, pH 5.2 for cabbage and 0.1 M acetate buffer, pH 5.0 for puffballs, respectively). The final volume was made to 5.0 mL. After incubation, for 20 min at 10 °C, the polymer was precipitated by adjusting the pH to 7.0 using 3 M NaOH [30]. The suspension was centrifuged at 12,000 × *g* for 5 min at 10 °C. The precipitate was washed with respective buffer till no enzyme activity was determined in the washings. Bound chitinase activity was calculated by the difference of initial activity added to chitosan solution and the activities recovered in supernatant and washings. The bound activity was later eluted by incubating the chitosan precipitate (having bound enzymes) with 3.0 mL of 2.0 M $MgCl_2$ prepared in the respective assay buffers (after dissolving $MgCl_2$, the pH had to be adjusted back to the assay buffer pH [30]). $MgCl_2$ at this concentration interfered with enzyme assays and was removed by dialysis, by dialysing against the respective assay buffer before determining the recovered enzyme activities.

2.2.10. Preparation of PEG–salt two-phase system

Stock solutions of 44% (w/v) PEG 6000 and 20% (w/v) dipotassium hydrogen orthophosphate (K_2HPO_4) were prepared in distilled water and pH was adjusted to 5.5. The concentrations of the two-phase components are given as % (w/v). Aqueous two-phase systems used were composed of 22% PEG and 10% K_2HPO_4, respectively. After vortexing for a minute, two distinct phases were formed within 5 min at 25 °C. Crude extracts of chitinases were added to the system. The volumes of these extracts were 330, 385 and 500 μL from *N. crassa*, cabbage and puffballs, respectively.

Binding of chitinases from *N. crassa*, cabbage and puffballs with chitosan as macroaffinity ligand in PEG–salt aqueous two-phase system. Aqueous two-phase separation using the PEG–salt system was used after incorporating 0.5% (w/v) chitosan solution. Extracts/homogenates containing chitinase activity from *N. crassa*, cabbage and puffballs (15, 27 and 35 U, respectively) were added separately to the aqueous systems. During the purification work, unclarified crude extracts/homogenates were used. In such cases, PEG and salt phases were separated by an interface consisting of cell debris and other insoluble matter. After vortexing, the phases were separated. The volume of the total system, before adding the crude extracts/homogenates, was 4.0 mL (without chitosan) and 5.0 mL (with chitosan), respectively. Chitosan distribution was restricted to the PEG-phase with less than 4% (of the initially added amount) going to the bottom-phase. Chitosan concentration in the two phases was estimated by phenol sulfuric acid method [33]. The top-phase containing the enzyme bound chitosan was removed with a pipette after 20 min. Chitosan was precipitated by adjusting the pH to 7.0, using 3 M NaOH [30]. The precipitate was centrifuged at 12,000 × *g* for 5 min at 10 °C. The supernatant and subsequent washings with buffer (till no enzyme activity was detected in the washings) were collected. Bound chitinase was calculated by the difference of initial activity and activity recovered in supernatant and washings. The bound activity was later eluted by incubating the chitosan precipitate (having bound enzyme) with 2.0 M $MgCl_2$ prepared in the respective assay buffers (just like the case of affinity precipitation experiments). $MgCl_2$ was removed by dialysis by dialysing against respective assay buffer before determining the recovered enzyme activity.

2.2.11. Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with 12% acrylamide was performed following the procedure of Hames [34] using Hoefer Mighty Small II; SE 250 Mini-Vertical Gel Electrophoresis Unit (Amersham Pharmacia Biotech, USA). The molecular mass markers consisted of mixtures prepared from a standard kit, which was obtained from Sigma (St. Louis, MO, USA). The standard kit included lysozyme (M_r 14,300), β-lactoglobulin (M_r 18,400), trypsinogen (M_r 24,000), albumin egg (M_r 45,000), albumin bovine (M_r 66,000) and phosphorylase *b* (M_r 97,400).

3. Results and discussion

N. crassa, cabbage and puffballs are well known sources of chitinases [26,28,29]. In order to establish assay conditions with chitin as the substrate, the pH optima (6.5, 5.2 and 5.0) and temperature optima (40, 45 and 30) of chitinases from *N. crassa*, cabbage and puffballs, respectively, were determined. These were found to be similar to those reported with chitin (in case of *N. crassa* and puffballs) [26,29] and ethylene glycol chitin (in case of cabbage) [28] as substrate. These assay conditions (pH and temperature) were used for monitoring purifications.

Chitosan is structurally very similar to chitin, it is obtained by partial deacetylation of *N*-acetylglucosamine moeities present in the latter. Many of the chitinases also hydrolyze chitosan. Hence, before using chitosan as a macroaffinity ligand, it was necessary to check whether it is degraded by chitinases during the process time and conditions. It was found that no detectable (by reducing group estimation with dini-

trosalicylic acid reagent) degradation of chitosan took place during 1 h (process time for both affinity precipitation as well as two-phase extraction) at 10 °C.

3.1. Affinity precipitation

Tables 1–3 constitute the purification tables from *N. crassa*, cabbage and puffballs, respectively, by affinity precipitation with chitosan. In each case, 82% or more of initial activity bound to chitosan and precipitated along with it. In each case, more than 80% activity was recovered by elution of the enzyme from chitosan. The dissociation of the enzyme from chitosan was carried out by adding $MgCl_2$ solution. Here, $MgCl_2$ obviously did not work on the basis of providing high ionic strength alone since chitosan did bind to the enzyme in the presence of phosphate buffer in the two-phase system. It has been pointed out that $MgCl_2$ is effective in dissociating antigen–antibody complexes because it simultaneously acts via high ionic strength, chaotropic effects and reduced pH (the lower pH also facilitated the dissociation) [35]. The fold purifications were 27, 15 and 30 for *N. crassa*, cabbage and puffballs, respectively. In each case, the purified enzyme gave a single band on SDS–PAGE.

3.2. Purification of chitinases by two-phase affinity extractions

Fig. 1a–c shows the variation in extent of activity bound and eluted (to chitosan in PEG–salt two-phase systems) with the different starting amounts of chitinase activities (*N. crassa* (2.5–45 U), cabbage (5–70 U) and puffballs (5–65 U)). The chitinase extracts introduced to aqueous two phases system were in the range of 70–1500 μL. As number of enzyme molecules increase, they start occupying available affinity sites on the incorporated chitosan molecules. As the extent of occupancy increases, the "crowding effect" prevents easy access to incoming enzyme molecules. Also, the available affinity sites are lot less. This gets reflected in the "approach to saturation" phase with decreased extent of binding of initial activity load. About 15, 27, and 35 U (the volumes of these crude extracts were 330, 385 and 500 μL, respectively) as starting load were found to be optimum. The elution throughout was above 80% of the bound activity.

Fig. 2 shows that incorporation of 0.5% (w/v) chitosan was adequate for binding the *N. crassa* chitinase activity. Increasing the concentration beyond this did not further enhance the percentage binding. Hence, 0.5% (w/v) chitosan was incorporated while carrying *N. crassa* chitinase purification by aqueous two-phase affinity extraction. In fact, 0.5% (w/v) chitosan was also found out to be adequate in the case of cabbage and puffballs chitinases as well. Fig. 3 indicates the eluted chitinase activities (*N. crassa*) and purification folds with different concentrations of $MgCl_2$.

Table 4 is the enzyme purification table for chitinase from *N. crassa*, in this case incorporation of chitosan resulted in

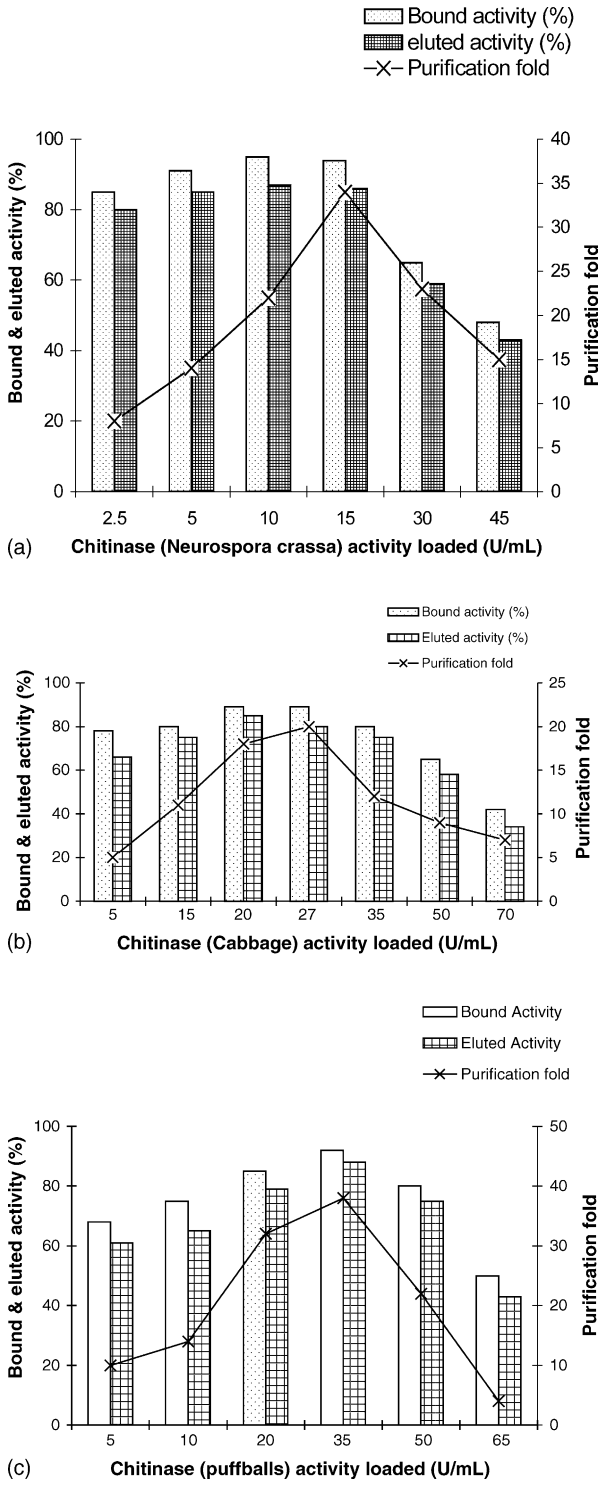

Fig. 1. (a–c) Effect of chitinase activities (*Neurospora crassa* (2.5–45 U), cabbage (5–70 U) and puffballs (5–65 U)) loaded on the binding, elution and purification folds. The binding of chitinase to 0.5% (w/v) chitosan as macroaffinity ligand was carried out as described in Section 2.2, using aqueous two-phase system. For the optimization of enzyme units, unclarified crude extracts were used. Bound chitinase activity was calculated by the difference of initial activity loaded and the activities of the supernatant and washing.

Table 1
Purification of chitinase from *Neurospora crassa* by affinity precipitation with 0.5% (w/v) chitosan solution

Step	Activity (U)	Protein (μg)	Specific activity (U mg^{-1})	Yield (%)	Fold purification
Crude	15.0	3000	5.0	100.0	1.0
Supernatant + wash	1.6	2100	0.8	11.0	0.2
Elution	12.8	95	135	85.0	27

Purification was done as described under Section 2.2 using clarified crude extract. The elution of the bound activity was carried out using 3.0 mL of 2.0 M $MgCl_2$ at 10 °C for 20 min. The activity was determined after extensive dialysis of $MgCl_2$. All the experiments were performed in duplicate and the difference in the readings in the duplicates was less than 5%.

Table 2
Purification of chitinase from cabbage by affinity precipitation with 0.5% (w/v) chitosan solution

Step	Activity (U)	Protein (μg)	Specific activity (U mg^{-1})	Yield (%)	Fold purification
Crude	27	463	58.3	100.0	1
Supernatant + wash	5	349	14.3	18.5	0.2
Elution	22	25	880	82	15

Purification was done as described under Section 2.2 using clarified crude extract The elution of the bound activity was carried out using 3.0 mL of 2.0 M $MgCl_2$ at 10 °C for 20 min. The activity was determined after extensive dialysis of $MgCl_2$. All the experiments were performed in duplicate and the difference in the readings in the duplicates was less than 5%.

Table 3
Purification of chitinase from puffballs by affinity precipitation using 0.5% (w/v) chitosan

Step	Activity (U)	Protein (μg)	Specific activity (U mg^{-1})	Yield (%)	Fold purification
Crude	35	1100	32	100	1
Supernatant + wash	3.2	700	5	9	–
Elution	31.5	33	960	90	30

Purification was done as described under Section 2.2 using clarified crude extract. The elution of the bound activity was carried out using 3.0 mL of 2.0 M $MgCl_2$ at 10 °C for 20 min. The chitinase activity was determined after extensive dialysis of $MgCl_2$. All the experiments were performed in duplicate and the difference in the readings in the duplicates was less than ±5%.

drastic increase in partitioning of chitinases in PEG-phase. This is understandable since 96% (w/v) chitosan was present in PEG-phase. In all the three cases (*N. crassa*, cabbage and puffballs), not more than 6% (w/v) chitinases remained in the lower salt-phase. In fact, presence of chitosan also decreased the activity loss due to chitinases activity remaining associated with cell debris etc. and forming the interfacial insoluble mass.

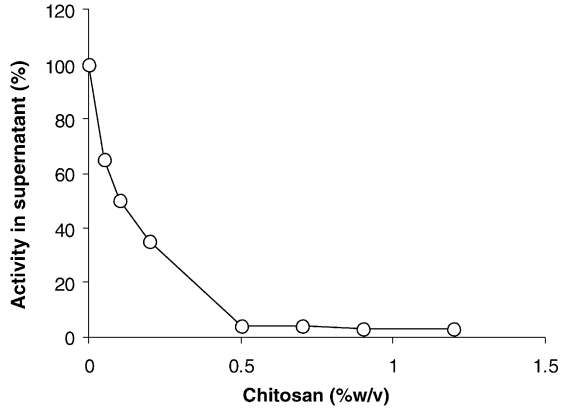

Fig. 2. Binding of chitinases from *Neurospora crassa* extract to different concentrations of chitosan. Different aliquots (such that final concentrations were 0.05, 0.1, 0.2, 0.5, 0.7, 0.9 and 1.1% (w/v), respectively) from 2% (w/v) chitosan solution were added to the aqueous two-phase system, after which 1.0 mL enzyme solution (containing 15 U) was incorporated. Later the aqueous two-phase system was vortexed and separated as mentioned in Section 2.2. The chitinase activity was determined in supernatant using chitin as substrate to determine the amount of bound activity.

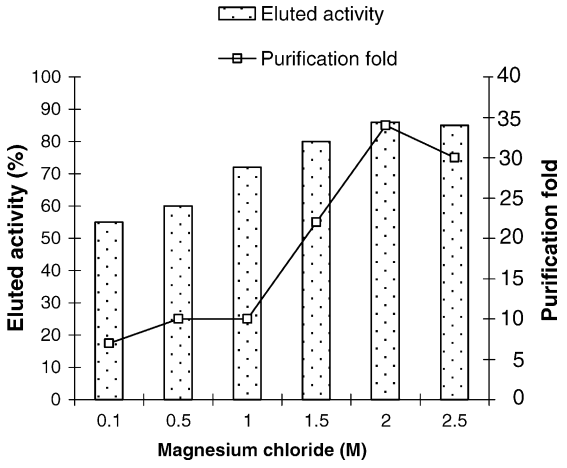

Fig. 3. Effect of different concentrations of $MgCl_2$ on elution and purification folds of *Neurospora crassa* chitinase using aqueous two-phase system. The activity initially added to the polymer is taken to be 100%. Controls confirmed the interference of $MgCl_2$ in the determination of enzyme activity. All the experiments were done in duplicates and the difference in the results in duplicates was less than 5%.

Table 4
Purification of chitinase from *Neurospora crassa* using PEG–chitosan salt two-phase system

Steps	Activity (U)	Protein (μg)	Specific activity (U mg^{-1})	Yield (%)	Fold purification
	15.0[a]	3000[a]	5.0[a]	100.0[a]	1.0[a]
Lower-phase (salt)					
No chitosan	5.1	1200	4.3	34.0	1.0
+Chitosan	0.9	1400	0.6	6.0	–
Interface					
No chitosan	3.0	800	3.8	20.0	1.0
+Chitosan	0.4	700	1.0	3.0	–
Upper-phase (PEG)					
No chitosan	6.2	500	12.4	41.0	2.5
+Chitosan (supernatant and washing)	0.7	300	2.3	5.0	1.0
Elution	12.9	75	172	86.0	34

Purification was done as described under Section 2.2 using unclarified crude extract. The elution of the bound activity was carried out using 3.0 mL of 2.0 M $MgCl_2$ at 10 °C for 20 min. The activity was determined after extensive dialysis of $MgCl_2$. All the experiments were performed in duplicate and the difference in the readings in the duplicates was less than 5%.
[a] Crude extract.

Use of reversibly soluble–insoluble macroaffinity ligand like chitosan allows to recover PEG-phase since chitosan and chitosan bound protein can be separated by increasing the pH to 7. The enzyme can be recovered from this precipitate by $MgCl_2$. In the case of *N. crassa*, 34-fold purification and 86% recovery of the chitinase activity could be obtained. Similar results were obtained with cabbage and puffballs. The cabbage enzyme was purified 20-fold with 80% activity recovery and puffball enzyme could be purified 38-folds with 88% recovery of the chitinase activity. The purified enzyme gave a single band on SDS–PAGE with molecular mass of 30,000 for cabbage (Fig. 4). The single bands were also obtained for purified chitinases from *N. crassa* and puffballs, agreeing with the earlier reported values of molecular mass of chitinases from these sources [26,29].

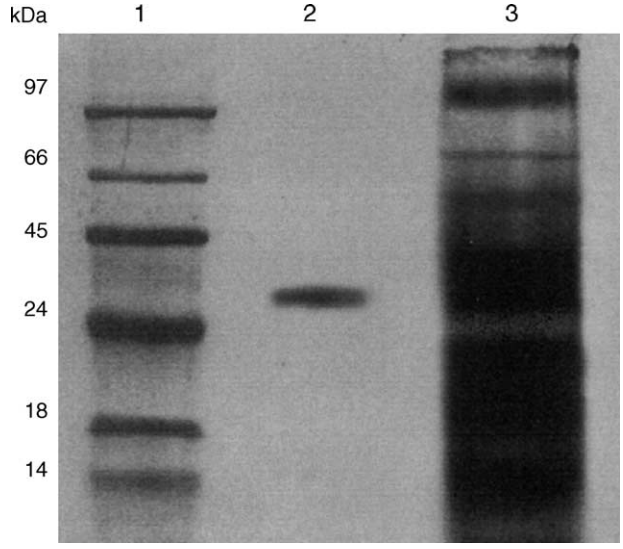

Fig. 4. SDS–PAGE patterns of cabbage chitinase. Lanes: 1 = marker proteins; 2 = purified preparation; and 3 = crude cabbage chitinase.

4. Conclusion

It is worthwhile to compare the fold purifications and activity recoveries obtained for chitinases from *N. crassa*, cabbage and puffballs with affinity precipitation and aqueous two-phase affinity system as two different purification strategies. Although, activity recoveries obtained were similar in all the three cases (*N. crassa*, cabbage and puffballs) with both techniques, two-phase affinity extraction consistently gave higher fold purifications as compared to affinity precipitation. This is understandable since in the format used here, two-phase affinity extraction is inclusive of some of the features of affinity precipitation. Moreover, two-phase affinity extraction allows one to deal with crude extracts/homogenates directly without preclarification/centrifugation. A major constraint of two-phase affinity extraction has been the cost of polymeric phases. Use of PEG–salt (instead of PEG–dextran) as two-phase system and removal of target protein along with chitosan would allow the reuse of PEG-phase. While we have not tried recycling of chitosan, we expect results similar to those of Senstad and Mattiasson [24]. Thus, it may be possible to recover chitosan to a significant level but some replenishment (10–20 %) would be required in each cycle. If purity and preclarification costs are not major issues, affinity precipitation itself can be used for obtaining chitinases as this technique itself gave a single band on SDS–PAGE. The results described here show that two-phase affinity extraction with chitosan as a macroaffinity ligand may become an economical, efficient and scalable approach for obtaining chitinases from diverse sources.

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References

[1] M.V. Deshpande, J. Sci. Ind. Res. 45 (1986) 277.
[2] J.P. Chen, K.C. Chang, J. Chem. Technol. Biotechnol. 60 (1994) 133.
[3] A.V. Ilyina, V.E. Tikhonov, V.P. Varlamov, L.A. Radigina, N.Y. Tatarinova, I.A. Yamskov, Biotechnol. Appl. Biochem. 21 (1995) 139.
[4] A.V. Ilyina, V.P. Varlamov, V.E. Tikhonov, I.A. Yamskov, V.A. Davankov, Biotechnol. Appl. Biochem. 19 (1994) 199.
[5] F. Shahidi, J.K.V. Arachchi, Y.-J. Jeon, Trends Food Sci. Technol. 10 (1999) 37.
[6] R.A.A. Muzzarelli, Chitin, Pergamon Press, Oxford, UK, 1977.
[7] R. Vaidya, S. Roy, S. Macmil, S. Gandhi, P. Vyas, H.S. Chhatpar, Biotechnol. Lett. 25 (2003) 715.
[8] B. Bhusan, G.S. Hoondal, Biotechnol. Lett. 20 (1998) 157.
[9] G. Xia, C. Jin, J. Zhon, S. Yang, S. Zhang, C. Jin, Eur. J. Biochem. 268 (2001) 4079.
[10] I. Kawachi, T. Fujida, M. Ujita, Y. Ishii, K. Yamagishi, H. Sato, T. Funaguma, A. Hara, J. Biosci. Biotechnol. 92 (2001) 544.
[11] C.-J. Woo, H.-D. Park, Biotechnol. Lett. 25 (2004) 409.
[12] V.E. Tikhonov, L.A. Radigina, I.A. Yamskov, N.D. Gulyaeva, A.V. Ilyina, M.V. Anisimova, V.P. Varlamov, N.Y. Tatarinova, Enzyme Microb. Technol. 22 (1998) 82.
[13] J.G. Huddleston, A. Lyddiatt, in: M. Verrall (Ed.), Downstream Processing of Natural Products, Wiley, Chichester, 1996, p. 53.
[14] M. Kamihira, R. Kaul, B. Mattiasson, Biotechnol. Bioeng. 40 (1992) 1381.
[15] D. Guoqiang, R. Kaul, B. Mattiasson, J. Chromatogr. A 668 (1994) 145.
[16] S. Teotia, M.N. Gupta, J. Chromatogr. A 923 (2001) 275.
[17] S. Teotia, M.N. Gupta, Protein Expr. Purif. 22 (2001) 484.
[18] S. Teotia, M.N. Gupta, J. Chromatogr. A 1025 (2004) 297.
[19] A. Sharma, S. Sharma, M.N. Gupta, Protein Expr. Purif. 18 (2001) 111.
[20] S. Teotia, R. Lata, S.K. Khare, M.N. Gupta, J. Mol. Recognit. 14 (2001) 295.
[21] S. Teotia, S.K. Khare, M.N. Gupta, Enzyme Microb. Technol. 28 (2001) 792.
[22] S. Sharma, A. Sharma, M.N. Gupta, Bioseparation 9 (2000) 93.
[23] I. Roy, M.N. Gupta, J. Chromatogr. A 998 (2003) 103.
[24] C. Senstad, B. Mattiasson, Biotechnol. Bioeng. 34 (1989) 389.
[25] R. Tyagi, A. Kumar, M. Sardar, S. Kumar, M.N. Gupta, Isol. Purif. 2 (1996) 217.
[26] A. Arroyo-Begovich, Methods Enzymol. 161 (1988) 471.
[27] P. Bridge, in: S. Doonam (Ed.), Methods in Molecular Biology, vol. 59, Humana Press, Totowa, NJ, 1996.
[28] C.-T. Chang, H.-F. Lo, C.-J. Wu, H.-J. Sung, Biochem. Int. 28 (1992) 707.
[29] J.P. Zikakis, J.E. Castle, Methods Enzymol. 161 (1988) 490.
[30] M.N. Gupta, B. Mattiasson, in: G. Street (Ed.), Highly Selective Separations in Biotechnology, Chapman and Hall, London, 1994, p. 7.
[31] G.L. Miller, Anal. Chem. 31 (1959) 426.
[32] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[33] C.H.W. Hirs, Methods Enzymol. 11 (1967) 411.
[34] B.D. Hames, in: B.D. Hames, D. Rickwood (Eds.), Gel Electrophoresis of Proteins: A Practical Approach, IRL Press, Oxford, 1986, p. 1.
[35] M.L. Yarmush, K.P. Antonsen, S. Sundaram, D.M. Yarmush, Biotechnol. Prog. 8 (1992) 168.