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κ-Carrageenan as a new smart macroaffinity ligand for the purification of pullulanase

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Abstract

κ-Carrageenan is a polysaccharide from red seaweed which gets precipitated by K^+ ions and dissolves again in water. This smart, K^+-responsive polymer was found to selectively bind pullulanase activity from *Bacillus acidopullulyticus*. Gel filtration on Sephadex G-200 showed the formation of the polymer–pullulanase complex at the pre-precipitation stage. On the other hand, phospholipase D, an enzyme which did not co-precipitate with κ-carrageenan, did not form any complex with the polymer. Thus, K^+ ions could be used to selectively precipitate the pullulanase activity. Then, 92% enzyme activity could be eluted with 1 *M* maltose solution. The single step protocol resulted in 50-fold purification, with a single band on sodium dodecylsulfate–polyacrylamide gel electrophoresis.
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1. Introduction

Affinity precipitation involves selective precipitation of the target protein by a smart macroaffinity ligand [1–3]. The latter is generally obtained by covalently linking a suitable affinity ligand to a smart polymer which can be made soluble or insoluble in response to a suitable stimulus [4]. The covalent linking can be carried out by well-established covalent coupling protocols for obtaining affinity media [5]. The technique, like other non-chromatographic techniques, is expected to show limited resolution. However, the selectivity of affinity interactions has

ensured that in many cases, the purified protein shows a single band on sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [6,7]. Thus, for many industrial applications, affinity precipitation is capable of giving adequate purification.

There are two main factors, which limit the wider and/or industrial applications of this technique: (i) the cost of linking the affinity ligand to the polymer. Also, covalent coupling uses harsh chemicals and this comes in the way of permitting the use of the resultant affinity macroligand for purification of target proteins meant for food and pharmaceutical purposes by the regulatory agencies; (ii) the availability of smart or stimulus-sensitive polymer. While in principle, many polymers have been described which are responsive to pH, temperature, light and magnetism [4,8], very few are available commercially. The latter include Eudragit [6,9,10], alginate

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[7,11,12] and chitosan [13,14], which have been used for affinity precipitation.

In recent years, our laboratory has been focusing on the possibility of using naturally occurring polysaccharides as stand alone smart macroaffinity ligands [7,12,14]. In this work, we show that κ-carrageenan can be used for designing smart macroaffinity ligand. Furthermore, we show that this nontoxic and economical polymer shows inherent affinity towards pullulanase and can be used to purify this enzyme by affinity precipitation. Pullulanase is a part of US$ 600 billion global market for starch-degrading enzymes [15].

2. Materials and method

2.1. Materials

κ-Carrageenan (Catalog No. 22048) was purchased from Fluka Biochemika (St. Louis, MO, USA). Sephadex G-200 was obtained from Sigma (St. Louis, MO, USA). Promozyme, a commercial preparation of pullulanase from *Bacillus acidopullulyticus*, Pectinex Ultra SP-L, a commercial preparation of pectinase and BAN 480L, a commercial preparation of α-amylase, were products of Novo Nordisk, Denmark and were purchased from Arun and Co., Mumbai, India. Peanuts were purchased from the local market and phospholipase D was isolated according to the procedure described in Ref. [12]. All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Estimation of enzyme activity and amount of protein

Activity of pullulanase was estimated using pullulan as the substrate [16]. One enzyme unit liberates 1 μmol of reducing sugar (calculated as glucose) per min at 40 °C and pH 5.0. The activity of pectinase was estimated according to the method described in Ref. [17]. One enzyme unit liberates 1 μmol of reducing sugar (calculated as galacturonic acid) per min at 30 °C and pH 5.0. α-Amylase activity was estimated as described in Ref. [18]. One enzyme unit liberates 1 μmol of reducing sugar (calculated as maltose) per min at 25 °C and pH 5.2. In all the

cases, the amount of reducing sugar produced was estimated by dinitrosalicylic acid method [19]. The activity of phospholipase D was estimated titrimetrically as described in Ref. [12], using a pHstat titrator (Metrohm model 718 STAT titrino) connected to an exchange unit and a magnetic stirrer. One unit of enzyme activity is defined as the amount of enzyme which liberates 1 μmol of acid from soybean lecithin per min at 25 °C at assay pH. Protein content was estimated by the dye binding method [20] using bovine serum albumin as the standard protein. Suitable controls were run to check for the non-interference of κ-carrageenan in protein and enzyme activity measurements.

2.2.2. Preparation of κ-carrageenan solution

κ-Carrageenan (0.3 g) was added slowly to 30 ml of distilled water with stirring to get a 1% solution of the polymer. The amount of κ-carrageenan was estimated by the phenol–sulphuric acid method [21].

2.2.3. Precipitation of κ-carrageenan

κ-Carrageenan (1%) was mixed with different concentrations of KCl (stock solution 5%) and the final volume made up to 3 ml with 0.05 M acetate buffer, pH 5.0. The solution was vortexed and incubated for 30 min at the desired temperature. The solution was then centrifuged (8000 g, 10 min, 25 °C) and the precipitate obtained was dissolved in 3 ml of 0.05 M acetate buffer, pH 5.0. Both the supernatant and precipitate obtained were checked for κ-carrageenan. In case of precipitation of the polymer in the presence of enzymes, the total volume of the mixture was maintained at 3 ml and precipitation was carried out with 0.3% KCl at 37 °C. It was found that after precipitation had occurred (at 37 °C), the centrifugation could be carried out at either 37 °C itself or 25 °C; in either case, only 9% polymer was detected in the supernatant.

2.2.4. Purification of pullulanase by affinity precipitation with κ-carrageenan

Pullulanase (0.2 ml, containing 4705 U ml^{-1}) was added to κ-carrageenan solution (0.9 ml, 1%). The final volume made up to 3 ml with 0.05 M acetate buffer, pH 5.0. Thus, the concentration of κ-carrageenan in the mixture was 0.3%. The solution was

incubated at 25 °C for 1 h. Precipitation of the enzyme-bound polymer complex was initiated by the addition of 125 μl of KCl (5%). The solution was incubated at 37 °C for 30 min and then centrifuged as described earlier. The precipitate was washed with 0.2% KCl (in 0.05 *M* acetate buffer, pH 5.0) till no enzyme activity could be detected in the washings. The precipitate was then dissolved in 2 ml of 1 *M* maltose (in 0.05 *M* acetate buffer, pH 5.0) and kept at 4 °C for 4 h. This was followed by precipitation of the polymer by the addition of 0.2% KCl, followed by centrifugation. The supernatant obtained was dialyzed overnight against the assay buffer and checked for enzyme activity.

2.2.5. Gel electrophoresis

SDS–PAGE of the samples was performed according to Hames [22] using a Bangalore Genei, electrophoresis unit and standard molecular mass markers (Bangalore Genei, Bangalore, India) using 10% gel.

3. Results and discussion

Carrageenan is a naturally occurring family of carbohydrates extracted from red seaweed. It is a high molecular mass polysaccharide made up of repeating galactose units and 3,6-anhydrogalactose, both are either sulphated or non-sulphated. The monomers are joined by alternating α-1,3 and β-1,4 glycosidic linkages. The three kinds of carrageenan—kappa, iota and lambda, differ in the number and position of the ester sulphate groups. These structural differences result in different temperatures at which carrageenan becomes soluble in the presence of K^+ [23]. Among these, κ-carrageenan has already been used for whole cell entrapment [24]. Thus, this polymer appeared to show considerable promise as a novel carrier for designing smart macroaffinity ligands.

Fig. 1 shows precipitation of various concentrations of κ-carrageenan at 37 °C by 0.2% KCl. κ-Carrageenan, with a concentration of 0.3%, showed 76% precipitation. To ensure optimum precipitation conditions for 0.3% κ-carrageenan, precipitation at different temperatures and KCl concentrations were tried (Fig. 2). Thus, 0.3% κ-carrageenan, at 37 °C, showed optimum precipitation with 0.2% KCl.
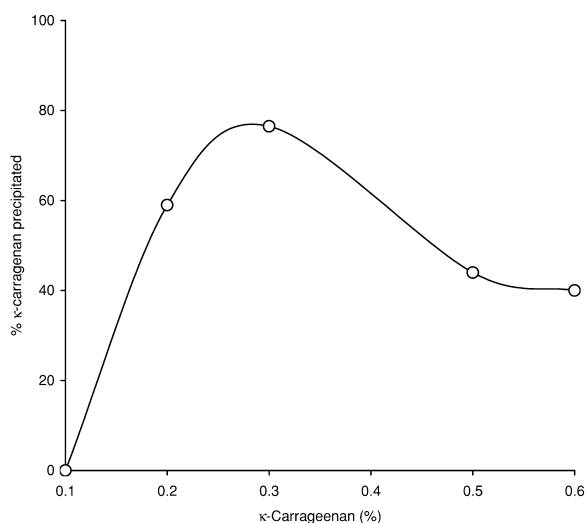

Fig. 1. Effect of concentration of κ-carrageenan on its precipitation behaviour. Various volumes of κ-carrageenan (stock solution of 1%) were taken and the final volume made up to 3 ml with distilled water. Precipitation was initiated by the addition of 0.2% KCl at 37 °C and the solution was then centrifuged (8000 *g*, 10 min) at 25 °C (±0.2). The precipitate was dissolved in 3 ml of distilled water and the supernatant and precipitate were checked for polysaccharide by phenol–sulphuric acid.
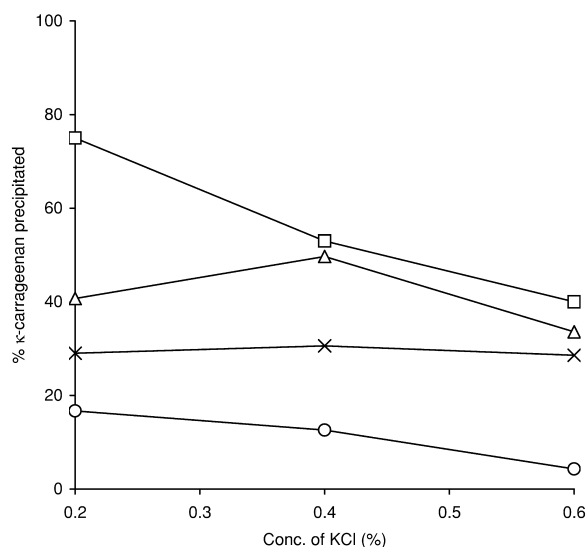

Fig. 2. Precipitation of 0.3% κ-carrageenan at 20 °C (○), 37 °C (□), 45 °C (△) and 50 °C (×). κ-Carrageenan (0.3%) was precipitated with different concentrations of KCl (stock solution 5%) and at the desired temperature for 30 min, after making up the total volume, as described in legend to Fig. 1.

Table 1
Precipitation of enzymes with 0.3% κ-carrageenan

Enzyme	Source	Load (U)	Bound activity (%)	U bound mg^{-1} polymer
α-Amylase	*Bacillus amyloliquefaciens*	1317	44	65
Pectinase	*Aspergillus niger*	728	94	76
Pullulanase	*Bacillus acidopullulyticus*	941	94	98
Phospholipase D	Peanut	17.8	0	–

Precipitation of proteins was carried out as described in the text. The bound activity is calculated by subtracting the enzyme activity obtained in washings from the activity initially loaded, which is assumed to be 100%.

Table 1 shows precipitation of four different enzymes along with κ-carrageenan under these conditions. The choice of the four enzymes was not purely arbitrary. We have earlier found that alginate, a polysaccharide consisting of guluronic and mannuronic acid residues, selectively binds these four enzymes [3,11,12,25]. Thus, it was thought worthwhile to check whether κ-carrageenan, another anionic polysaccharide, will show similar molecular recognition. As the data in Table 1 show, κ-carrageenan behaves somewhat differently from alginate in its selectivity towards these enzymes. Pullulanase (an enzyme which hydrolyzes α-1,6 glycosidic bonds in starch) and pectinase (an enzyme which hydrolyzes pectin), showed high amount of affinity towards the polymer. As a suitable affinity eluent, viz. maltose, exists for pullulanase, it was decided to work with this enzyme. Maltose is a known competitive inhibitor of the enzyme [25] and was expected to elute the enzyme out of the affinity matrix, viz. κ-carrageenan.

It is worth noting that κ-carrageenan showed higher precipitation (91%) with pectinase and pullulanase, as compared to its precipitation in the absence of these enzymes (76%, Fig. 2). Thus, formation of enzyme–polymer complex to an adequate extent seems to facilitate precipitation. This is somewhat similar to the experience with alginate

[11,26]. No κ-carrageenan was detected in the washings. The formation of such complexes and the kinetics of growth of such complexes during the pre-precipitation stages have been studied by light scattering [27]. In the present work, simple gel filtration on Sephadex G-200 (column with bed volume of 245 ml) was used to investigate the formation of the enzyme–polymer complex at the pre-precipitation stage. It was found that when κ-carrageenan was mixed with pullulanase, the polymer and the enzyme co-eluted within the void volume (70 ml). The polymer and the enzyme, when loaded alone, eluted much later, at elution volumes of 82.5 ml and 130 ml, respectively. When phospholipase D was used in place of pullulanase, no complex of κ-carrageenan with the enzyme could be detected and the enzyme as well as the polymer eluted at the same position when loaded alone.

This is in agreement with the observation (Table 1) that phospholipase D did not precipitate along with κ-carrageenan. The gel filtration also established clearly that in the precipitation of the three enzymes with κ-carrageenan (Table 1), K$^+$ merely aided the precipitation of the polymer and had no role in the complex formation between the polymer and the enzyme.

Table 2 is the purification table for pullulanase. About 91% of the polymer is precipitated along with

Table 2
Purification of pullulanase by affinity precipitation with κ-carrageenan

Steps	Activity (U)	Protein (mg)	Yield (%)	Specific activity (U mg^{-1})	Fold purification
Crude	1001	3.8	100	263	1
Wash	80	2.4	8	33	–
Eluate	916	0.07	92	13086	50

Details of the protocol are described in the text.

the enzyme. The enzyme-bound polymer is dissolved in 1 *M* maltose. The dissolution occurs since the concentration of KCl decreases and also because sugars are known to help in the dispersion of κ-carrageenan (FMC Biopolymer brochure on Marine Colloids Carrageenan). On addition of KCl after dissociation of the enzyme from the polymer (so that the final concentration of KCl in solution is 0.2%), 93% of the latter is precipitated, thus separating it from the purified pullulanase. It is seen that 92% enzyme activity can be eluted with ~50-fold purification. Co-precipitation of contaminating proteins is known to occur in these cases [28]. Table 2 shows that only about 37% protein precipitates along with the polymer (63% protein is recovered in the supernatant and washings). The selectivity also operates at the elution stage. Recovery of less than 2% of the bound protein shows the selectivity of the eluent, viz. maltose, for this enzyme. The purified enzyme shows a single band on SDS–PAGE, with an estimated molecular mass of 100 000 Da (Fig. 3). The specific activity, fold-purification and molecular mass ob-

tained are similar to what are reported for a purified pullulanase preparation [25].

Pullulanase is an industrially important enzyme. Thus, the simple and efficient purification protocol described here should help in lower production costs for the enzyme. At a more general level, the results with κ-carrageenan once again confirm our belief that polysaccharides show unusual and unexpected biological affinity towards enzymes [3,29]. κ-Carrageenan is a non-toxic and fairly inexpensive polymer which is widely used in food industries. Thus, its use is compatible with the end use of the purified enzyme in feed, food and even pharmaceutical industries. κ-Carrageenan has an advantage over alginate, another metal ion-responsive smart polymer. Use of Ca^{2+} in the case of the latter necessitates the addition of EDTA for dissociating Ca–alginate precipitate. It also rules out working with phosphate or citrate buffers. On the other hand, simply adding distilled water can dissolve precipitates of K^+ and κ-carrageenan and one can use all the common buffers like phosphate and citrate. κ-Carrageenan has free hydroxyl groups and hence suitable coupling methods (utilizing cyanogen bromide, epichlorohydrin, etc. [5]) exist for linking it to any suitable affinity ligand. Thus it can act as a general smart carrier for designing smart macroaffinity ligand for any target enzyme/protein. While this is so in principle, one needs to evaluate this experimentally with various systems. As the presence of a target enzyme improves the extent of precipitation, it is not unlikely that the approach will work in at least a few cases. Recently, we have shown that alginate and Eudragit can also be used in two-phase affinity extraction to facilitate direct purification without precipitation of target enzymes from crude suspensions [30,31]. Similar studies are in progress with κ-carrageenan. We are also investigating whether κ-carrageenan, like Eudragit S-100, can be used to purify enzyme via macroaffinity ligand facilitated three phase partitioning [32].
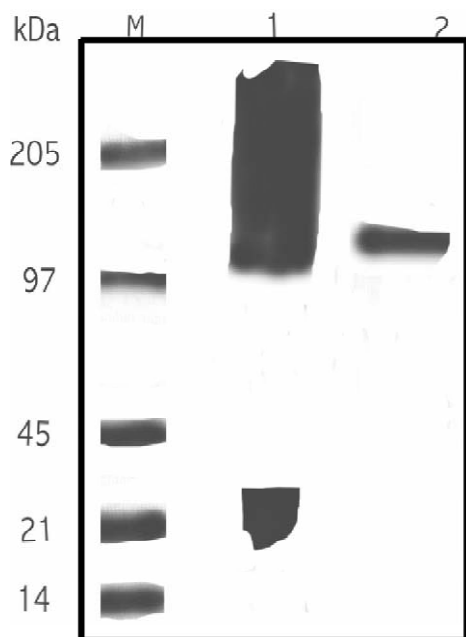

Fig. 3. SDS–PAGE of purified enzyme. Lanes: 1, crude enzyme (20 μg); 2, purified enzyme (25 μg); M, marker proteins (20 μg). The gel was stained with Coomassie Brilliant Blue R-250 for 60 min and then destained in 40% methanol and 10% acetic acid.

Acknowledgements

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