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# High performance liquid chromatography of *Bacillus circulans* peptidoglutaminase for laboratory and industrial uses

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**Abstract**

Gel permeation and anion-exchange chromatography were used to develop methodology for large-scale production of peptidoglutaminase (PGase) from *Bacillus circulans* cell extract. Sample load, flow-rate and elution profiles were optimized to obtain a highly active DNA-free PGase preparation in high yield. PGase was fractionated into I and II and purified up to 1430-fold for enzymatic determination of glutamine, molecular cloning and protein deamidation research. PGases were also separated directly from *B. circulans* extract (20–30 mg) in one peak with an 8-fold purification on a 43-ml anion-exchange column at 2 cm/min in 35–40 min. More than 65% of the cell extract proteins were eluted after the PGase peak and contained all the nucleic acids. This method appears to meet the requirements of purity, yield, speed and other economic aspects for successful production of PGase for potential modification of food proteins in industrial reactors.

## 1. Introduction

Deamidation of proteins is the hydrolysis of amide groups to acidic groups and is carried out to increase the net negative charges on proteins. This lowers the isoelectric point of the protein and increases solubility, emulsifying capacity and other important functional properties in mildly acidic food systems [1]. An enzymatic approach to protein deamidation is preferred over chemical approaches because of the mild conditions, safety, speed and selectivity of enzymatic reactions. Transglutaminase, protease and peptidoglutaminase (PGase) are the only enzymes reported in the literature for protein deamidation. PGase seems to be the most feasible for industrial application because of the prohibitive cost and other potentially severe problems for the food processor that may be associated with the other two enzymes [2]. PGase hydrolyzes the γ-amides of L-glutamine (Gln) residues in peptides and proteins and is used to modify their structures. PGase can be separated into two fractions (I and II) by multiple steps of conventional chromatography [3]. Fraction I hydrolyzes the amide groups on C-terminal Gln residues and II hydrolyzes the amide groups on the other Gln residues.

It is crucial for the successful use of PGase for protein modification in industry to develop large-scale methodology for producing food-grade PGase. Processes for the production and/or use of PGase must be cost-effective, and amounts of DNA and other extraneous materials must be less than that specified by the US Food and Drug Administration. Bioreactor methods were developed in this laboratory for the recovery and multiple use of PGase in industrial settings [2]. However, large-scale purification methods such as precipitation, gel adsorption and ultrafiltration

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could not fractionate PGases into I and II and were ineffective in increasing PGase specific activity by more than 2-fold [2,4]. Therefore, there is a need for a better preparative method to obtain large quantities of PGase from *Bacillus circulans* cell extract in high yields that are free of all the nucleic acids. Most enzymes manufactured for industrial use need to be stable and inexpensive but do not need to be highly purified [5]. Cost, desired purity, speed, practicality, stability and yield are among the main issues that should be addressed in method development for preparative chromatography. This work reports (1) the successful application of HPLC methods to isolation and fractionation of PGase on a laboratory scale for use in research and (2) the suitable values of variables for fast, practical, efficient large-scale HPLC method of PGase for industrial uses.

## 2. Experimental[1]

### 2.1. Preparation and assay of PGase

*B. circulans* (ATCC 21590) cells were grown, harvested and extracted with 0.01 *M* phosphate buffer, pH 8.0, as previously reported [4]. Cell extracts and eluates were evaluated for PGase I and II activities in 0.05 *M* phosphate buffer, pH 7.0 at 30°C, using the synthetic substrates carbobenzoxy (CBZ)-L-glutamine and *tert.*-butoxycarbonyl (Boc)-L-glutaminyl-L-proline (Peptides International, Louisville, KY, USA), respectively. The specific activities of PGase I and PGase II in cell extract were 9–14 and 11–15 μmol $mg^{-1}$ $h^{-1}$, respectively.

### 2.2. The HPLC system

The preparative chromatography system Delta Prep 3000 from Waters Assoc. (Milford, MA, USA) was used for analytical and preparative HPLC separation of *B. circulans* cell extracts. Aliquots of PGase in 0.02 or 0.05 *M* sodium phosphate buffer, pH 8.0, were filtered through a 0.45-μm Millex-HV Filter (Millipore, Bedford, MA, USA) before their injection. Elution was monitored at 280 nm by a Model 481 Lambda-Max spectrophotometer detector connected to a Waters 745B data module for the integration of the proportions of various eluted peaks. Fractions were collected using a Foxy fractionator (ISCO, Lincoln, NE, USA). HPLC-grade reagents and solvents were used throughout this investigation.

### 2.3. Analytical quaternary methylamine (QM) anion-exchange HPLC

Preliminary ion-exchange separation of PGase was performed on an analytical column Accell QMA, 15 cm × 3.9 mm I.D. (Waters). The protein load was 1.0 mg protein in 20 μl 0.02 *M* phosphate buffer, pH 8.0. Eluent used in gradient separation was 0.05 *M* sodium phosphate buffer (pH 8.0) and 0.1–0.8 *M* KCl at a flow-rate of 0.5 ml/min.

### 2.4. Semipreparative QM and DEAE anion-exchange separation

Ion-exchange separation of PGase was performed on a steel column (15 cm × 19 mm I.D.) packed with Accell Plus QMA medium from Waters and a 15 cm × 21.5 mm I.D. prepacked column Protein-PAK DEAE 5 PW (Waters). The protein load was 2–30 mg in 1–3 ml 0.02 *M* phosphate buffer, pH 8.0. The gradient separation eluent was 0.05 *M* sodium phosphate buffer (pH 8.0) and 0.1–0.8 *M* KCl. The flow-rate was 1.5–10.0 ml/min.

### 2.5. Semipreparative gel permeation

A prepacked column of cross-linked methacrylate gel (Ultrahydrogel Linear, 30 cm × 7.8 mm I.D. from Waters) with an effective fractionation range from $M_r$ 2000 to $7 \cdot 10^6$ was used. Aliquots of PGase sample (20 μl) in 0.05 *M* sodium phosphate buffer (pH 8.0) were injected

[1] Commercial firms are mentioned in this publication solely to provide specific information. Mention of a company does not constitute a guarantee or warranty of its products by the US Department of Agriculture nor an endorsement by the Department over products of other companies not mentioned.

then eluted with 0.05 *M* sodium phosphate buffer (pH 8.0). The flow-rate was 0.3 ml/min and protein load was 2–30 mg. The high protein load run was repeated 12 times and the corresponding fractions from each run that contained PGases were pooled, dialyzed and then lyophilized, after excluding the fractions eluted in the first and last 6 min. This protein (0.1 mg) was reinjected and separated again under same conditions used with the other gel permeation separations. Ferritin, catalase, aldolase, bovine albumin, hen albumin and chymotrypsinogen A were used for column calibration. Their apparent molecular masses were 540, 240, 158, 67, 45 and $25 \cdot 10^3$, respectively, according to the manufacturer, Sigma (St. Louis, MO, USA).

### 2.6. *Purification and fractionation of PGase*

The pooled PGase peak from the multiple injection to the Ultrahydrogel Linear column was injected into the Accell Plus QMA column (2 mg), then eluted with 0.05 *M* sodium phosphate buffer (pH 8.0) and KCl gradient at 1.5 ml/min to fractionate PGase into I and II. Fractions containing either PGases I or PGase II activities were dialyzed, freeze-dried and stored at −20°C until used.

### 2.7. *PGase deamidation of protein hydrolysates*

The protein hydrolysates were previously prepared [6] by hydrolyzing soy protein, casein and gluten to 5, 10, 15 and 20% peptide bond hydrolysis with Alcalase (Novo Lab., Wilton, CT, USA). The extent of deamidation, using PGases I and II was calculated as the ratio of ammonia released enzymatically to the total amide content of the protein hydrolysate, multiplied by 100.

### 2.8. *Analyses of DNA and protein fractions*

Protein contents of PGase preparations and eluates were measured by the BCA method of Smith et al. [7]. The nucleic acid concentration in PGase preparations and eluates was measured by the spectrophotometric method of Schleif and Wensink [8]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 8–25% polyacrylamide gels using the PhastSystem (Pharmacia/LKB, Uppsala, Sweden) and a pH 8.9, 0.125 *M* Tris–borate buffer containing 0.1% SDS, as originally described by Koenig et al. [9].

### 2.9. *Statistical analysis of variance*

Multi-factor analysis of variance [10] of variables was performed using the software package and the procedures of Statgraphics (Rockville, MD, USA).

## 3. Results and discussion

### 3.1. *Analytical separation and scale-up of anion-exchange chromatography*

In earlier work, PGase separation into I and II was achieved by sequential precipitation with streptomycin sulfate (to remove nucleic acids) and ammonium sulfate followed by chromatography on Sephadex G-200, DEAE-Sephadex and hydroxyapatite [3]. In developing the separation method here, newly introduced, advanced HPLC tools were taken advantage of so that the PGase separation is carried out directly from the cell extract without any sample preparation or other fractionation techniques. Therefore, key interdependent experimental parameters such as mobile phase composition, pH, and ionic strength were investigated to fractionate PGase into I and II and obtain a fast, high-recovery separation. The method development for anion-exchange chromatography was first initiated by using an analytical column (15 cm × 3.5 mm I.D.) to assess the chromatographic performance of the anion exchanger Accell QMA. The separation was carried out at pH 8.0 using the KCl gradient program and other initial conditions based on the conventional separation on DEAE-Sephadex [3]. The proteins were separated into six major peaks (each contained more than 2% of the total protein) with the last three peaks, grouped to one main peak, containing 65% of the total protein (Fig. 1A). PGases were not separated into I and II as the preceding peak had 89% of

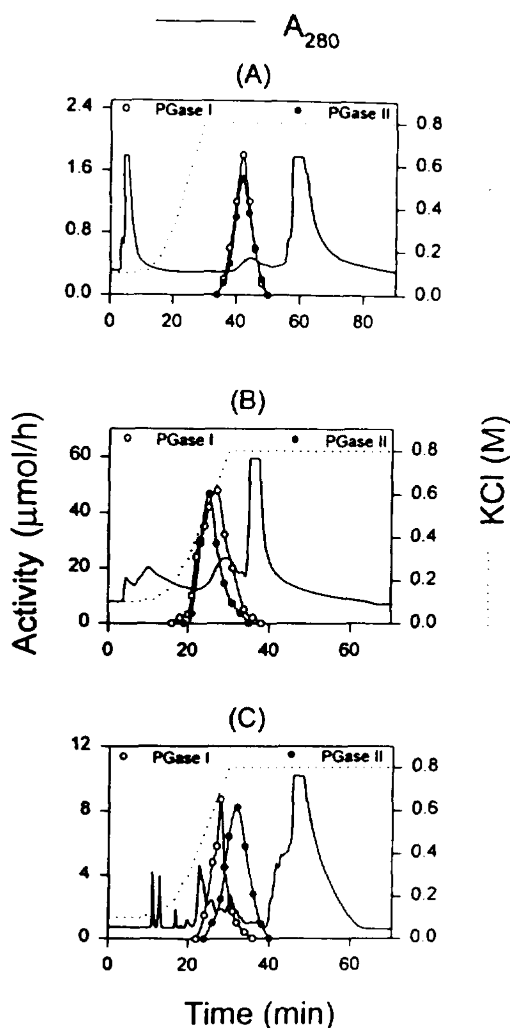


Fig. 1. Anion-exchange separation of *B. circulans* proteins using (A) QM anion-exchange (15 cm × 3.9 mm I.D.) column at 1.0 mg load and 0.5 ml/min. (B) 15 cm × 19 mm I.D. QM anion-exchange column (30 mg load) at 5.0 ml/min and (C) DEAE anion-exchange (15 cm × 21.5 mm I.D.) column at 5 mg load and 5 ml/min.

the activity units of both PGase I and II with 9-fold increase in their specific activities.

This separation was scaled-up to a semipreparative scale using a 15 cm × 19 mm I.D. column with the same bed length but about 30 times the volume of the analytical column. Scale-up of ion-exchange process is usually achieved by increasing the column diameter while maintaining the column bed height and linear flow-rate constant [11]. Chromatographic separation loads are usually scaled up a number of times equal to the ratio of their cross sectional areas. Here, the protein load was increased from 1 to 30 mg, a scale-up factor of 30 based on the volumes of the two columns. Ideally, runs are carried out at the same linear velocity (flow-rate in ml divided by the cross-section area of the column) to maintain an equivalent time frame for the small- and large-scale separations [12]. During scale-up, the flow-rate was changed from 0.5 ml/min (5.2 cm/min) to 5 ml/min (1.8 cm/min). The reduction of scale factor for the linear flow-rate was chosen to improve PGase resolution in an attempt to affect the separation of PGases I and II. To gain some increase in resolution, many chromatographic separations are scaled up at much lower flow-rates than those based on maintaining linear velocity [13]. The Accell QMA chromatography on the semipreparative column using 0.05 *M* phosphate buffer (pH 8.0) and a scaled-up KCl gradient (Fig. 1B) also gave six peaks of which the last one accounted for 69% of the total protein. PGases I and II were still eluted in one peak with 86% recovery and a 9-fold purification

Reducing the protein load gradually from 30 to 5 mg increased fold purification from 7 to 12 but was ineffective in fractionating PGase into I and II. A typical anion separation of the proteins from the extract of *B. circulans* cells is presented in Fig. 1C in which another column (15 cm × 21.5 I.D.) containing DEAE anion exchanger was used at a flow-rate of 5 ml/min and 5 mg load, with the optimal gradient profile developed for the QM anion-exchange runs. The PGase yield was 89% and the ion exchange gave 16 peaks, of which the last peak contained all the nucleic acids and accounted for 72% of the total protein. This peak was preceded by the peaks of the of PGase I and II. PGases were fractionated into I and II but there was still overlapping. The PGases were purified 13.6- and 11.4-fold, respectively, for PGases I and II with an average of 12.5-fold. In all the runs, separation efficiencies of the DEAE and Accell columns were comparable. In spite of that, the Accell column is preferred because it can be easily self-packed

and the support medium can be effectively regenerated for continuous use.

### 3.2. Effect of flow-rate and protein load on anion-exchange separation

Flow-rates and protein loads were varied to study their effects on the QM anion separation (Table 1). Analysis of variance indicated that both flow-rate and protein load significantly affected the purity of the PGase. The last peak of the chromatographs contained a large portion of the injected protein ranging from 65–80%. Protein content of this peak as a percentage of the total injected proteins was independent of the protein load but it declined significantly by increasing the flow-rate. Regardless of its protein content, this peak contained all the nucleic acids of the injected cell extract and thus they can be easily separated from the PGase peaks. Fig. 2 shows the anion separation using the QM column (A) and the DEAE column (B) at 3 ml/min and 10 mg protein load. Reducing protein load from 30 to 10 mg and flow-rate from 5 to 3 ml/min was still ineffective in ending overlapping in the fractionation of PGase into I and II. However, reducing the protein load injected into the anion column from 10 to 5 mg or less and the flow-rate from 3 to 1.5 ml/min resulted in separation of I and II after retention times of 74 and 93 min, respectively, with complete resolution of the two peaks (Fig. 2C). The PGases were purified 14.2- and 14.7-fold, respectively, and followed by the last peak that contained 78% of the protein. The separation of *B. circulans* cell extract with protein load of 20 mg and a flow-rate of 6 ml/min is shown in Fig. 2D. PGases I and II were not separated, but rather they eluted in one peak. The yield and the degree of purification of PGase were more than 85% and 8-fold, respectively and the separation was complete in 35–40 min. More than 65% of the protein was eluted last after the PGase peak, which contained all the nucleic acids that were in the injected sample. The conditions of this separation were the most suitable for scale-up applications since increasing flow-rates to more than 6 ml/min or loading to more than 30 mg led to the deterioration of the separations, particularly the fold purification of PGase peak.

Sample load is a key parameter influencing throughput since it has a dramatic effect on separation efficiency ($\alpha$), column efficiency, retention time and even peak shape [13]. Sample load is directly proportional to $\alpha$ and hence the easiness or difficulty of separation. Also, as sample load is increased, the effective plate count decreases until the efficiency reaches a point that the column is no longer useful for practical separation. Both the flow-rate and protein load were reduced to effect the fractionation of PGases into I and II and were also maximized to develop a large-scale method (Fig. 2). Flow-rate is the operational variable which most influences time. High flow-rates are possible with separation efficiencies, $\alpha$, of more than 2.0, while working within the constraints of acceptable back pressure and practical solvent handling capabilities. As $\alpha$ becomes smaller, the flow-rates may have to be decreased to maintain

Table 1
Fold purification of the PGase peaks separated on QM anion exchanger at various protein loads and flow-rates

| Flow-rate (ml/min) | Protein load (mg) | | | Multiple range analysis[a] | |
|---|---|---|---|---|---|
| | 5 | 10 | 20 | Mean ± standard error | Homogenous groups |
| 1.5 | 14.2 | 12.2 | 10.0 | 12.1 ± 0.2 | I |
| 3.0 | 11.9 | 9.9 | 9.2 | 10.3 ± 0.2 | II |
| 6.0 | 11.5 | 9.5 | 8.3 | 9.8 ± 0.2 | III |

[a] For fold purification by flow-rate. Group I is significantly different from group II and from group III, but groups II and III do not differ significantly from each other.

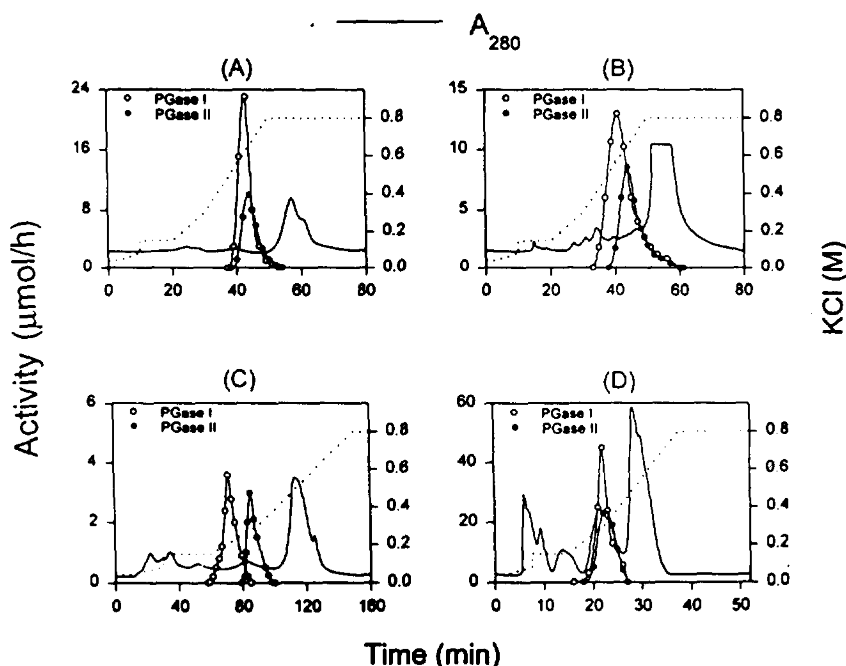


Fig. 2. Anion-exchange chromatography of *B. circulans* proteins on (A) QM column, (B) DEAE column (10 mg load at 3.0 ml/min for both runs), (C) QM anion exchanger at 1.5 ml/min with protein load of 5 mg, and (D) QM ion exchanger at 6.0 ml/min and 20 mg load of cell extract proteins.

the desired degree of resolution and solute recovery [13].

### 3.3. Gel permeation HPLC

The separation of the proteins from *B. circulans* (2 mg loads) on the Ultrahydrogel column is presented in Fig. 3A. Gel permeation gave 9 peaks, of which the first three were non-PGase proteins and accounted for 91% of the total protein with all the nucleic acids present in the third peak. The 4th peak had PGase I and II activities and only 0.4–0.6% of the protein. This resulted in a 250-fold increase of the specific activity of both PGases with 90% yield. The PGases were slightly separated into two overlapping peaks (Fig. 3A) allowing the estimation of their molecular masses. Estimated $M_r$ values were $180 \cdot 10^3$ and $220 \cdot 10^3$ for PGases I and II, respectively. Runs of 20–30 mg loads (Fig. 3B) gave only six overlapping peaks. Although the yield was basically as high as that of the runs of lower loads, the separation efficiency was inferior: the specific activity of the PGase peak increased only 60–100-fold and this peak contained about 20–35% of the nucleic acids. At these high protein loads, each of the PGases had a small tailing peak containing 13.5% of the total activity with an estimated $M_r$ of $90 \cdot 10^3$ and $110 \cdot 10^3$ for PGases I and II, respectively (Fig. 3B). These lower-$M_r$ species of PGases were evidently dissociated forms of the $180 \cdot 10^3$ or $220 \cdot 10^3$ PGases. Kikuchi and Sakaguchi [14] reported the $M_r$ of the non-dissociated PGases to be $200 \cdot 10^3$ and the PGase I and II to be $90 \cdot 10^3$ and $125 \cdot 10^3$, respectively, in dissociating conditions of 0.1 *M* KCl and pH 10.9. The pooled PGases of the 20–30 mg loads were reinjected into the gel permeation column. PGases were separated into two overlapping peaks (Fig. 3C) with retention times corresponding to estimated $M_r$ of $180 \cdot 10^3$ and $220 \cdot 10^3$ for PGases I and II, respectively. More than half of the injected proteins were removed during this run, doubling

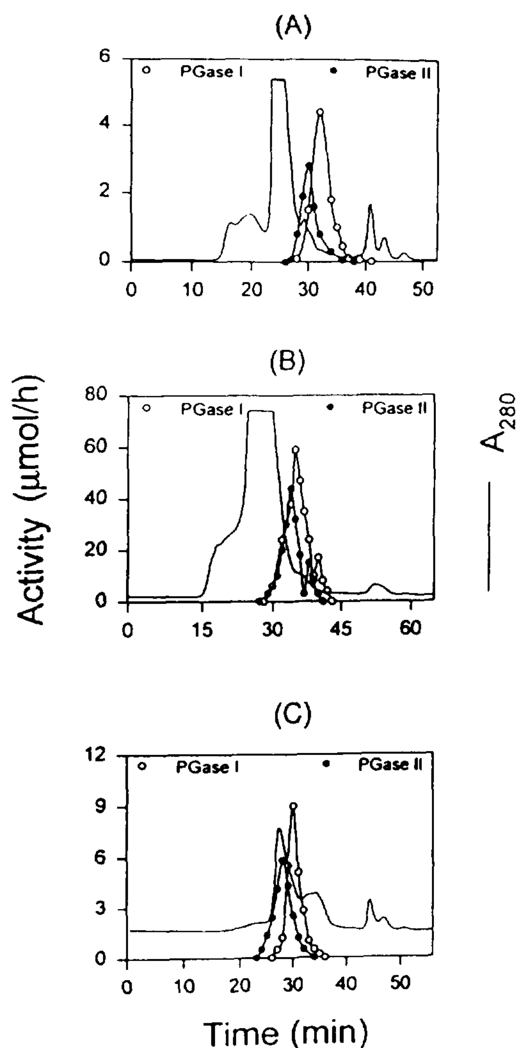


Fig. 3. Gel permeation of *B. circulans* proteins on acrylate gel (30 cm × 7.8 mm I.D.) at a flow-rate of 0.5 ml using 2 mg (A) and 20 mg (B) of cell extract proteins. Pooled active peaks (0.1 mg) from (B) were reinjected (C).

the fold purification of the PGase (I and II) preparation.

### 3.4. Role of the PGases in protein deamidation

PGase I and PGase II obtained by anion-exchange HPLC were used to hydrolyze the amide groups of the glutamine residues in three proteins with diverse structures. The deamidation of the hydrolysates of soy protein, casein and gluten using PGase I and PGase II is presented in Fig. 4. The total PGase deamidation of protein hydrolysates, the sum of % deamidation by PGases I and II, was equal to the percent deamidation previously obtained [2] using partially purified preparations of *B. circulans* cell extract, which were mixtures of PGases I and II. Here, over 52, 37 and 39% total protein deamidation was achieved for soy protein, casein and gluten, respectively, that had 20% peptide bond hydrolysis (20 DH). PGase I alone was responsible for 17, 21 and 42% of the deamidation of soy, casein and gluten at a DH range of 5–15 but dropped to 16, 17 and 31% at 20 DH. PGase II was responsible for the remainder of the deamidation. Gill et al. [15] found no deamidating activity of PGase I toward whey and casein proteins or their 4 DH hydrolysates. Here both enzymes (PGase I and II) were needed for the completion of the deamidation of proteins. Therefore, PGase preparations must contain both enzymes. Separation of PGase I from PGase II is not needed, providing that sufficient PGase I activity is present in the PGase I and II mixture.

### 3.5. Purification of PGases to homogeneity by gel permeation and anion HPLC

Proteins of *B. circulans* cell extracts were first subjected to gel permeation chromatography to obtain a PGase peak with a 60–100-fold increase in specific activity (20–30 mg loads). PGase was further fractionated on an Accell preparative column (2 mg loads) into I and II (Table 2). The average yield of PGase activity units was 80%. Specific activity of the PGase peak increased 1310- and 1540-fold for PGases I and II, respectively, as compared to the original cell extract (average 1425-fold). Kikuchi et al. [3] used conventional chromatography to obtain 13 and 21% of total activity of PGases I and II from *B. circulans* cell extract with 400- and 420-fold purification, respectively. The SDS-PAGE patterns of the PGase I and PGase II contained one band each with $M_r$ of $45 \cdot 10^3$ and $55 \cdot 10^3$, respectively, as estimated using protein markers (Fig. 5). It appears from these data and the gel

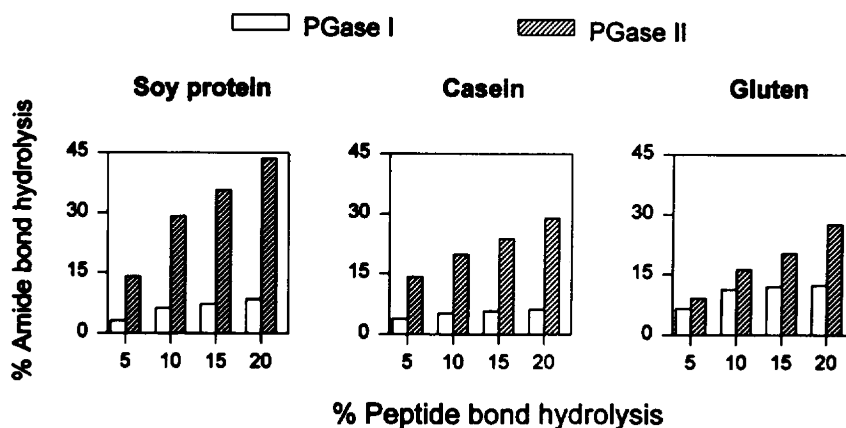


Fig. 4. Percent deamidation of protein hydrolysates effected by PGase I and PGase II, both purified from cell extract by anion chromatography.

permeation data that *B. circulans* PGases have $M_r$ of $180 \cdot 10^3$ and $220 \cdot 10^3$ that can be dissociated into $90 \cdot 10^3$ and $110 \cdot 10^3$ units, respectively. Also, dissociated PGases may have one intermolecular disulfide bond linking two subunits of $45 \cdot 10^3$ for PGase I and $55 \cdot 10^3$ for PGase II.

It appears that the purity of both PGases is sufficient to make them suitable for use in probing for cloning these enzyme. Re-engineering the PGases is necessary to increase their production during fermentation and the relative concentration of PGase II, responsible for the majority of protein deamidation (60–80%). Additionally, cloning would allow the production of these enzymes in a food grade microorganism such as *Bacillus subtilis* or *Saccharomyces cervisiae*. Further, using these purification methods on a laboratory scale are helpful in obtaining PGases for use in the determination of glutamine in proteins or peptides. The individual content of asparagine and glutamine, making up the total amide content in protein or protein hydrolysate can be determined by amino acid and amide analysis, liquid chromatography or by enzymatic methods [1]. Enzymatic methods, however, may be more reliable because of their selectivity and the liable nature of the amide group even at very mild experimental conditions. Purified PGases can be used in the determination of glutamine in peptides and proteins according to the method of Kikuchi [16].

Table 2
Deamidating activity and yield of *B. circulans* extract after HPLC purification

| Enzyme or HPLC method | PGase I | | | | | PGase II | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Protein (mg) | Total units[a] | Recovery (%) | Specific activity[b] | Fold purification | Protein (mg) | Total units[a] | Recovery (%) | Specific activity[b] | Fold purification |
| Cell extract | 344 | 3234 | 100 | 9.4 | – | 344 | 4919 | 100 | 14.3 | – |
| Gel permeation | 4.1 | 3007 | 93 | 734 | 78 | 3.7 | 4526 | 92 | 1257 | 88 |
| Ion exchange | 0.2 | 2587 | 80 | 12 317 | 17 | 0.16 | 3539 | 78 | 22 025 | 18 |

[a] One unit releases 1.0 μmol $NH_3$ per h.
[b] Units per mg protein.

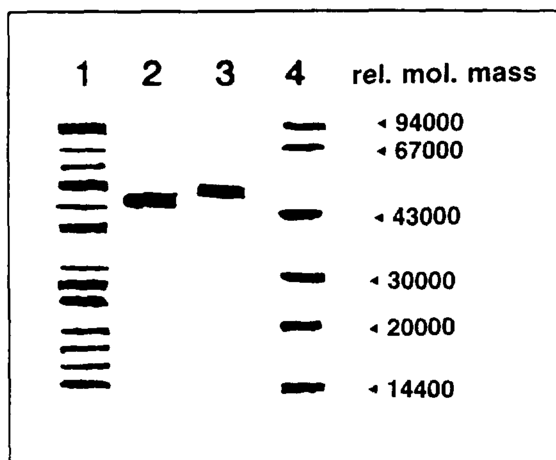


Fig. 5. SDS-PAGE of *B. circulans* cell extract (lane 1) and PGase I (lane 2) and PGase II (lane 3) both purified from cell extract by gel permeation and anion-exchange chromatography. Samples in lane 4 are the molecular mass markers.

### *3.6. Preparative separation method of PGase for industrial use*

Ion-exchange chromatography is widely used for downstream processing of significant proteins [11] but gel permeation fractionation of proteins is also considered a standard technique that can be scaled up to process level. The relatively high purity and the excellent resolution achieved with gel permeation were offset by the enzyme contamination with nucleic acids and the lack of speed for this method of separation. Protein overloading resulted in contamination of PGase preparations with DNA due to overlapping of the PGase peak with preceding peak that contained all the DNA. Because nucleic acids are heterogenous and contain molecules with widely different sizes, they can be eluted into more than one peak on gel permeation with late elution of the peak containing low molecular masses [17].

On the other hand, ion exchange can be of more practicable value in large-scale production of proteins. Since both PGases are needed for protein deamidation and their fractionation is not required, the conditions of PGase separation on the QM column using 20–30 mg protein at 6 ml/min were the most suitable for the scale-up process. In this method, PGase could be separated directly from *B. circulans* extract in one chromatographic run in less than 1 h with high purity and high yield. More than two-third of the injected protein was found in the last peak, which contained all the nucleic acids of the injected cell extract. Due to the high negative charges in DNA phosphate groups, anion-exchange separation can be the most effective technique for the removal of DNA contaminant from protein preparations [17,18]. Therefore, this purification program would have an impact on the economy of this large-scale technique since expensive chromatographic separations, chemical or enzymatic treatments for the removal of nucleic acids from *B. circulans* extracts are not needed. Furthermore, this anion separation method meets the requirements of purity, yield, speed, and the practical and economical aspects for successful large-scale production of the enzyme. Therefore, this suitable separation developed on this preparative unit is ready to be scaled-up directly using appropriate equipment.

## 4. Conclusions

(1) PGase can be isolated from *B. circulans* cell extract by preparative scale HPLC using either anion-exchange or gel permeation, but only the former yields an enzyme entirely free of nucleic acids. In gel permeation the nucleic acids elute before the PGase, resulting in some tailing, whereas in anion exchange the nucleic acids elute after the PGase.

(2) PGase can be separated cleanly into its component fractions, PGase I and PGase II, by anion-exchange HPLC if the protein load and flow-rate are both reduced. The SDS-PAGE patterns of the two PGases show subunits of $45 \cdot 10^3$ and $55 \cdot 10^3$, compared to $M_r$ of $180 \cdot 10^3$ and $220 \cdot 10^3$ for the undissociated enzymes.

(3) Both PGase I and PGase II are needed to match the extent of deamidation of typical protein hydrolysates achieved with the whole PGase, contrary to a literature report. For this application, the separation of the two PGases is probably unnecessary.

(4) Anion-exchange separation on preparative

HPLC unit was optimized to develope methodology for the potential production of kilogram quantities of PGase from *B. circulans* cell extract.

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