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Recombinant Enzymes - From Basic Science to Commercialization

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Chapter 12
Case Study: Recombinant Bromelain Downstream Processing

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Abstract This chapter presents details on the purification, formulation and drying of recombinant bromelain. The wide range of applications of recombinant bromelain has increased interest in finding viable purification techniques for large-scale production. An affinity chromatography technique was developed by Amid and co-workers (Expression, purification, and characterization of a recombinant stem bromelain from Ananas comosus, Process Biochem 46:2232–2239, 2011) to purify recombinant bromelain. However, this technique presented low recovery and small sample loading capacity and thus is not suitable as a purification tool in the large-scale production of recombinant bromelain. An aqueous two-phase system is one alternative method that we use to purify recombinant bromelain, as it reliable and easy to scale up and has a low cost. As part of avoiding cysteine degradation, spray drying the purified recombinant protein with maltodextrin as an excipient provides the possibility of preserving its activity and creating fine particles that are suitable for end-product application. The processes of the purification, formulation and spray drying of recombinant bromelain are explained briefly.

Keywords Back extraction · Continuous ultrasonication · Gibbs free energy · Inlet air temperature · Maltodextrin · PEG-rich phase · Salting-out · Immiscible

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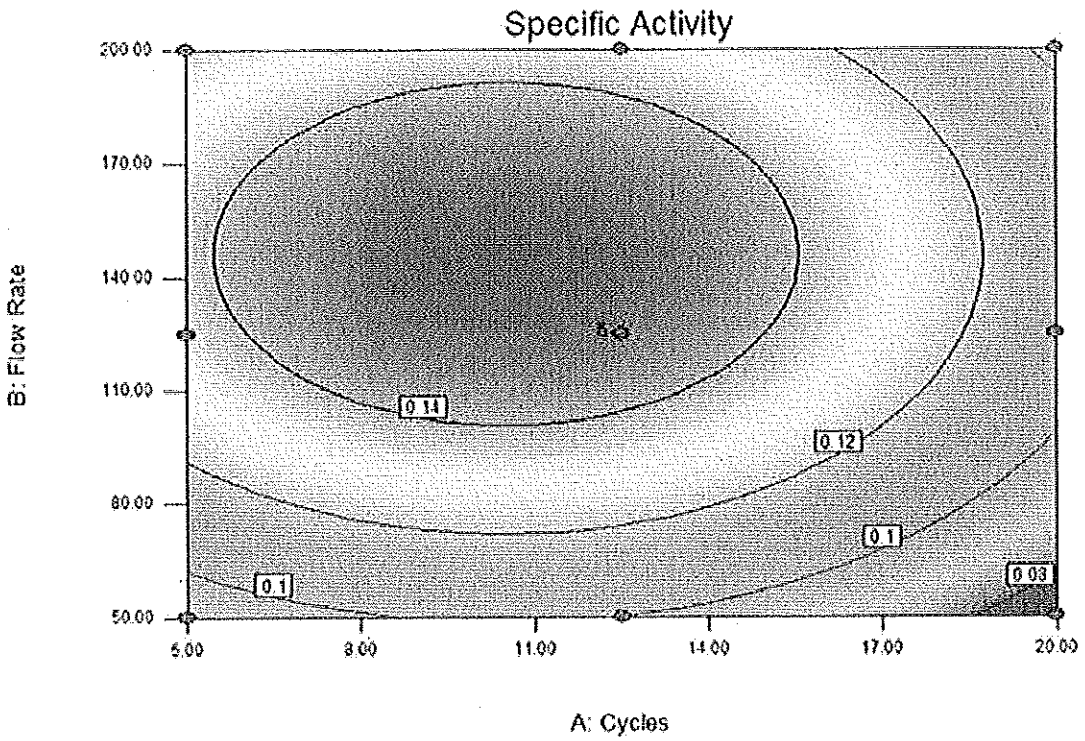

Fig. 12.1 Contour plot on the effect of cycles, *A* (passes), and flow rate, *B* (ml/min), on the specific activity, *Y* (U/mg-protein), of continuous cell disruption by ultrasonication

12.1 Cell Disruption by Continuous Ultrasonication

A normal cell disruption technique applied for large-scale processing is homogenization. However, the produced recombinant bromelain is heat sensitive, and heat generated during the homogenization process deactivates the enzyme, and thus, a continuous ultrasonication technique was used in our case. Figure 12.1 shows the effect of the flow rate and bursting cycles during the ultrasonication process on the specific activity of our recombinant bromelain product. These data suggest that a medium bursting cycle and medium flow rate provide the highest specific activity of recombinant bromelain. A high flow rate might not be able to disrupt most of the cells in the medium, whereas too high of a bursting cycle might reduce the specific activity of the enzyme.

12.2 Partial Purification of Recombinant Bromelain by Ammonium Sulfate Precipitation

Partial purification by ammonium sulfate precipitation was performed using ammonium salt powder as reported in Doonan [2] with a slight modification. At 50% (w/v) ammonium sulfate saturation and above, darker visible bands could be

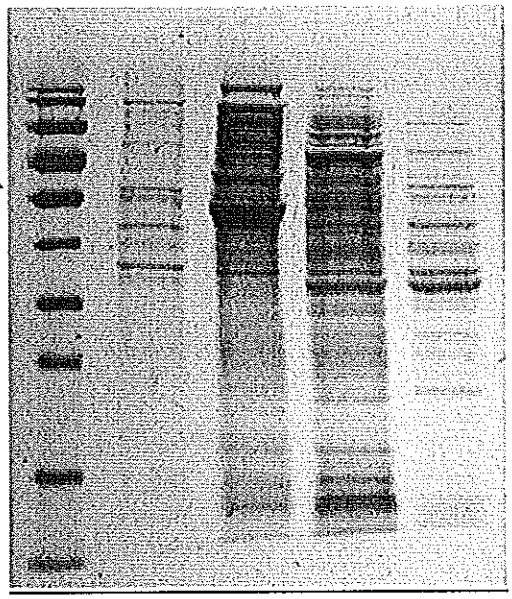

Fig. 12.2 Precipitate protein fraction under SDS-Page. Lanes: (*1*) a PageRuler® marker and (*2*) 25%, (*3*) 50%, (*4*) 75% and (*5*) 100% ammonium sulfate saturation

observed. Based on the previous report by Amid and co-workers [1], the band for recombinant bromelain is positioned at approximately 50 kDa (Fig. 12.2).

12.3 Purification of Recombinant Bromelain

The ATPS system involves the formation of two immiscible phases after the mixing of polyethylene glycol (PEG)-salt in an aqueous solution. In this work, we evaluate the performance of PEG8000/K_2HPO_4 in purifying recombinant bromelain using a two-step ATPS method: forward and back extraction. The factors affecting the partition behavior of recombinant bromelain, such as the pH, type of salt and concentration of PEG8000 and K_2HPO_4, were also investigated to elucidate the optimum conditions for purification of the enzyme. Figure 12.3 summarizes the ATPS extraction method.

12.3.1 Selection of the Salt Types

To identify a suitable salt for the purification of recombinant bromelain, several ATPSs were conducted using different salt types and amounts of K_2HPO_4, Na_2SO_4 and $(NH_4)_2SO_4$. A phase system containing 17% w/v PEG8000 and 20% w/v K_2HPO_4 provided the highest specific activity, 4.194084 unit/mg, as shown in Fig. 12.4, and this system was selected for further study. The amount of lysate r-bromelain used in this study was 20% (w/v). The ability of K_2HPO_4 to produce a higher salting-out effect led to an effective partition of recombinant bromelain in the PEG-rich top phase. This is because anions with higher valence, such as

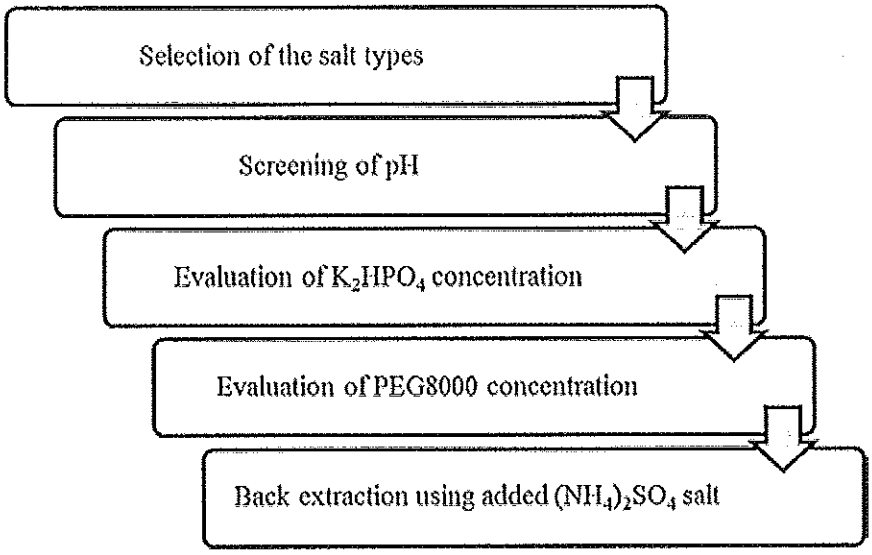

Fig. 12.3 Flow chart showing the ATPS extraction method

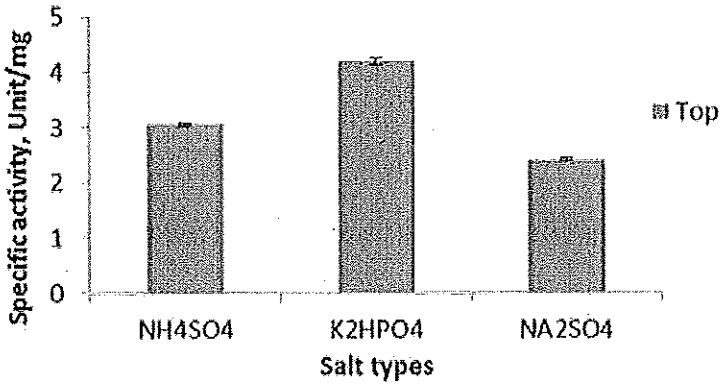

Fig. 12.4 Specific activity of recombinant bromelain with different salt types

K_2HPO_4, are better as salting out agents and decrease the amount of water available to hydrate polymers [3]. This findings follows the order of Gibbs free energy of hydration: $\Delta Ghyd(HPO_4^{2-}) = -1789$ kJ/mol > $\Delta Ghyd(SO_4^{2-}) = -1080$ kJ/mol. A superior salting-out ability is related to a higher negative value of Gibbs free energy of hydration [4, 5].

12.3.2 pH Screening

Figure 12.5 shows the recombinant bromelain specific activities versus pH, ranging from pH 6 to 9, with 17% w/v of PEG8000 and 20% w/v K_2HPO_4. The amount of lysate r-bromelain used in this study was 20% (w/v). The maximum recombinant bromelain activity occurred at pH 5 [1]; however, under ATPS conditions, it presented an apparent optimum specific activity at pH 8 of 8.433884 Unit/mg. Under alkaline conditions, PEG8000 appears to stabilize the biological activity of

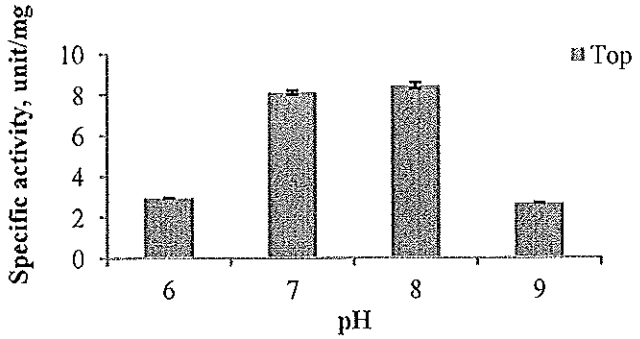

Fig. 12.5 Specific activity of recombinant bromelain at different pH values

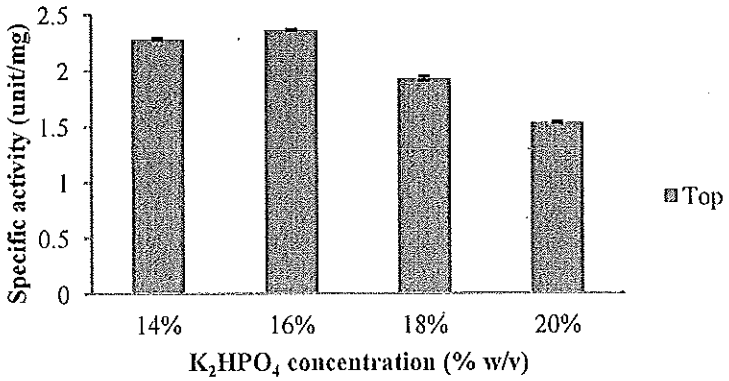

Fig. 12.6 Specific activity of recombinant bromelain at different K_2HPO_4 concentration

recombinant bromelain [6]. At higher pH values, the recombinant bromelain is negatively charged and will partition more into the PEG-rich phase [7].

12.3.3 Salt Concentration Screening

The effect of the K_2HPO_4 concentration on the recombinant bromelain partition is shown in Fig. 12.6. All of these experiments were performed at pH 8.0 with 17% w/v PEG8000 and different concentrations of K_2HPO_4, from 14 to 20% w/v. The amount of lysate r-bromelain used in this study was 20% (w/v). The maximum value of specific activity in the top phase is achieved at a concentration of 16% w/v, and thus, it was selected for further experiments. When the K_2HPO_4 concentration was higher than 16% w/v, the specific activity decreased due to the accumulation of recombinant bromelain in the interface of the bottom phase lower partition in the PEG-rich phase [7].

12.3.4 Screening of PEG8000 Concentration

The effect of the PEG8000 concentration, ranging from 12 to 20% w/v, was used to study the partitioning of recombinant bromelain, as shown in Fig. 12.7. During the

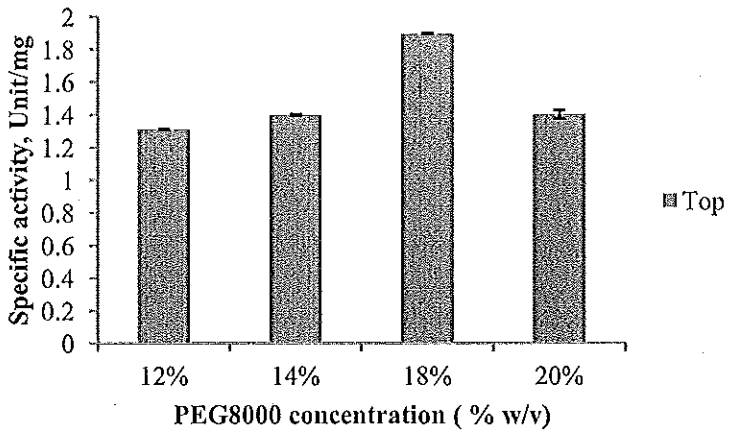

Fig. 12.7 Specific activity of recombinant bromelain at different PEG8000 concentration

experiment, the K_2HPO_4 concentration was maintained at 16% w/v, pH 8.0, with 20% (w/v) lysate r-bromelain. It was found that the highest specific activity was achieved at 18% w/v of PEG8000. It can be observed that the specific activity of recombinant bromelain is marginally increased from 12 to 16% w/v and then starts to decrease at 20% w/v. An increase in the polymer concentration causes the space available for recombinant bromelain to partition into the top phase to be reduced, and consequently, the protein tends to partition into the bottom phase [8].

12.3.5 Optimizing the ATPS Process Conditions

All of the values obtained from OFAT screening were used in the optimization of the ATPS process conditions. Three parameters were tested during the purification process optimizations: pH (6.5–8.5), K_2HPO_4 concentration (14–18% w/v), and PEG8000 concentration (16–20% w/v). The response surface plot graph in Fig. 12.8 indicates that the optimal process conditions for ATPS were 20% (w/v) PEG8000 and 18% (w/v) K_2HPO_4 solutions at pH 6.5. Next, these optimized process conditions were applied to the back extraction purification method.

12.3.6 Back Extraction ATPS Method

In the forward extraction, the chosen ATPS conditions included 20% (w/v) PEG8000 and 18% (w/v) K_2HPO_4 solutions (pH 6.5). Most of the recombinant bromelain partitioned to the top phase, and the bottom phase was discarded. A purification fold of 16.92 and yield of 20.29% were obtained in the forward extraction. In the second ATPS, fresh 18% (w/v) K_2HPO_4 and 8% (w/v) $(NH_4)_2SO_4$ solutions were added, and most of the recombinant bromelain was shifted to the top phase. After two-step extractions, the purification fold of the top phase was 3748.48, and the yield was 13.85%. The purification fold was higher compared with the single extraction method reported by Ketnawa and co-workers of (3.44-fold) [9]; and Ketnawa and

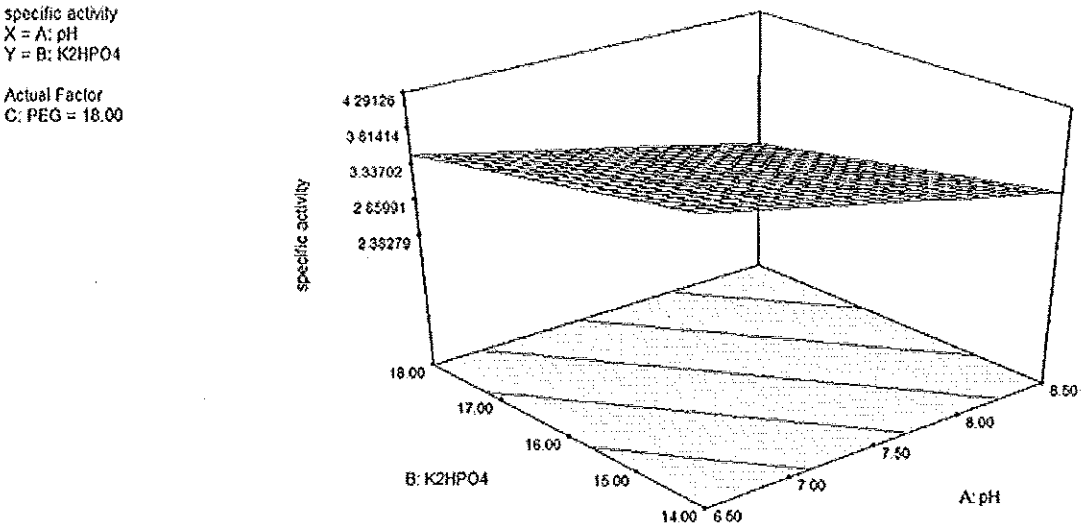

Fig. 12.8 Response surface plot of two variables pH and K_2HPO_4 concentration

Table 12.1 Purification table of recombinant bromelain in PEG8000/K_2HPO_4 ATPS by two-step extraction at pH 6.5 and 25 °C

		Total protein (mg/ml)	Enzyme activity (unit/ml)	Specific enzyme activity (unit/mg)	Purification fold	Yield (%)
	Crude	1.05	0.42	0.39	1	100
Forward extraction	Top phase	0.074	0.50	6.71	17.21	20.29
Back extraction	Top phase	0.015	22.70	1486.63	3811.87	13.85

co-workers (2.23-fold) [10]; Therefore, the two-step extraction method can facilitate the separation of recombinant bromelain from the PEG-rich phase in addition to helping achieve a high purification factor. Table 12.1 shows a summary of ATPS purification involving both the forward and back extraction method.

12.4 Formulation and Spray Drying of Recombinant Bromelain

Spray drying is commonly used in the production of pharmaceutical powders. In the present study, the top phase sample consisting of purified recombinant bromelain in a PEG-rich phase was collected before undergoing the spray drying process. The procedure was initiated by conducting the excipient screening. Five distinguished and commonly used excipients in the food and pharmaceutical industry were chosen. The main aim was to select a suitable excipient that could protect the recombinant enzyme solution from rapid dehydration during spray drying, thus retaining as much specific activity of the final spray dried recombinant bromelain as possible.

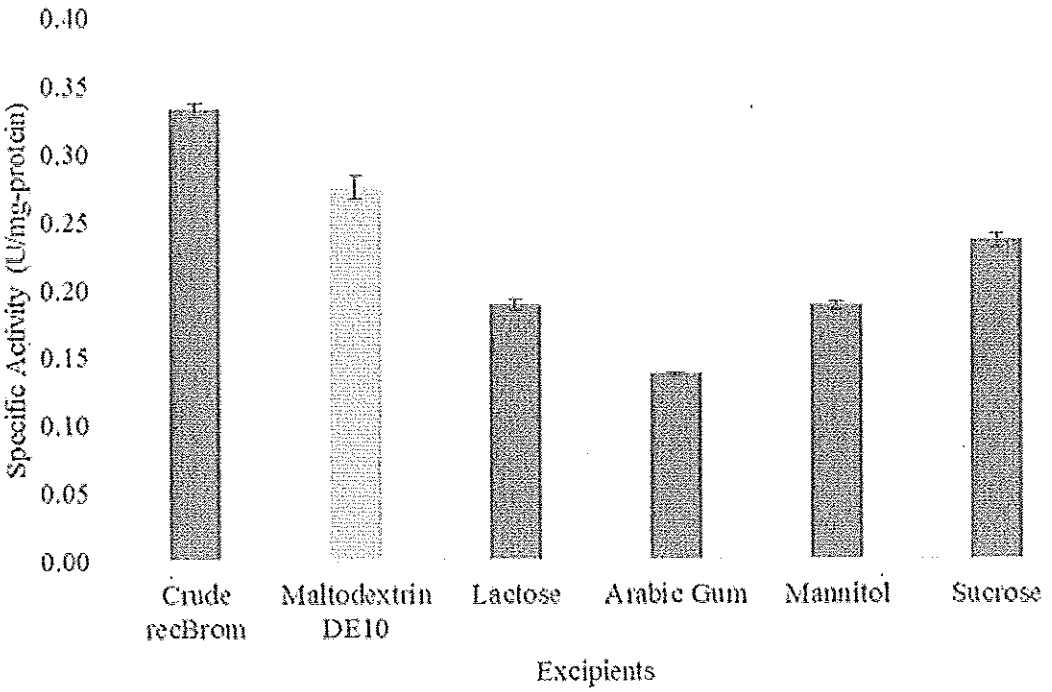

Fig. 12.9 Excipients screening with different types of excipients

The five selected excipients used for screening were Arabic gum, lactose, maltodextrin (dextrose equivalent, DE 10), mannitol and sucrose, which have been reported as binder agents, filler agents and microencapsulation agents for protecting bioactive compounds or active pharmaceutical compounds in many types of food and pharmaceutical products.

Figure 12.9 shows the clear graphical results of the excipients types used in this screening. The initial activity before the addition of excipients was 0.327±0.004 U/mg-protein. The lowest activity obtained was with Arabic gum with 0.137±0.024 U/mg-protein and 51.35% of activity lost. Thus, Arabic gum appears to have an inhibitory potential toward enzymatic activity [11]. Next, the suitable excipient was used in the formulation to produce dry recombinant bromelain through the spray drying technique.

The parameters chosen were the inlet air temperature, gas flow height and feed flow rate. The output response of the recombinant bromelain specific activity (U/mg-protein) varied from 0.020±0.002 to 0.112±0.001 U/mg-protein. Figure 12.10 shows the effect of the feed flow rate and inlet air temperature by a 3D response surface plot, and the highest point in the red region clearly shows the optimal range of response. It clearly shows that the increase of inlet temperature to a maximum with a decrease of gas flow height to a minimum reduces the specific activity of the spray dried recombinant bromelain. In the inlet temperature region of 110 to 130 °C, with a gas flow height from 40 to 50 mm, the specific activity is optimal. Devakate and co-workers [12] reported that desirable high activity was achieved using spray drying under low temperature conditions. Denaturation of protein might occur at high inlet temperature, which reduces the activity before the product is dried [12].

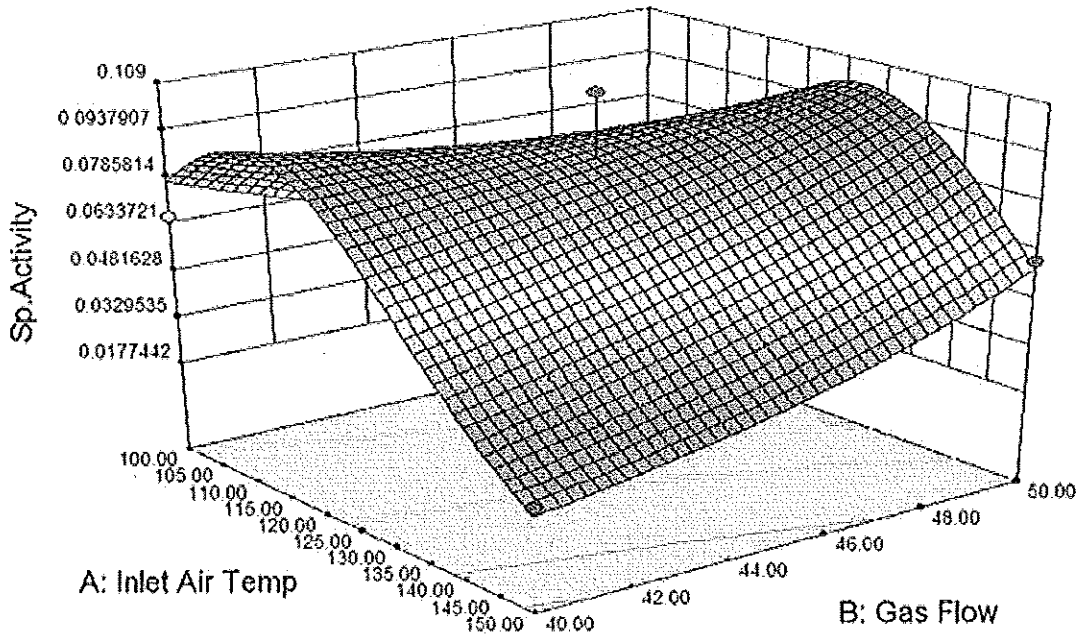

Fig. 12.10 3D surface plot of the effect of inlet air temperature, *A* (°C), and gas flow height, *B* (mm), toward specific activity (U/mg-protein) of spray dried recombinant bromelain

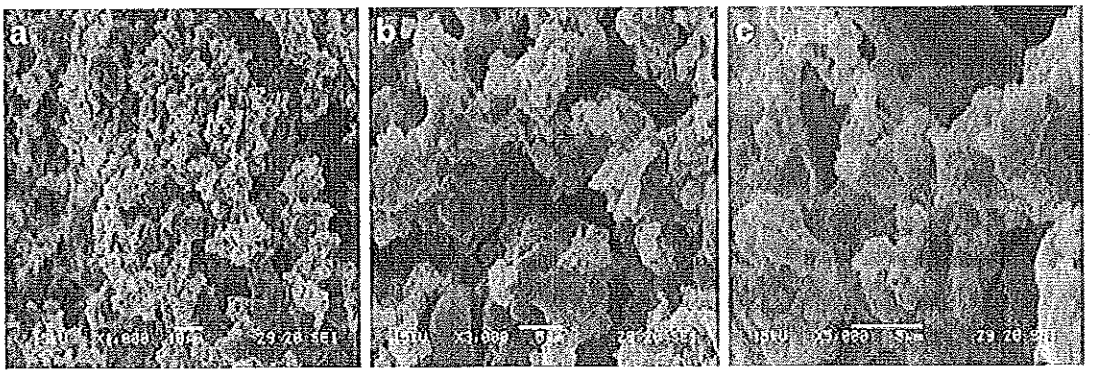

Fig. 12.11 SEM images of excipient-free spray dried recombinant bromelain. Three stages of magnification at (*A*) 1000X, (*B*) 3000X and (*C*) 5000X

The enzyme inactivation for bromelain reported by Devakate and co-workers [12] occurred at 65–70 °C, but with the addition of maltodextrin, the recombinant bromelain activity in this study was maintained at a high temperature of more than 120 °C with 50 % activity recovery

Dried recombinant bromelain was further subjected to a scanning electron micrograph (SEM) for particle morphology observation. Figure 12.11 shows the images captured at different magnifications, 1000X, 3000X and 5000X. The results in the figure indicate that without excipient-free recombinant bromelain powder, the particle shape is crust-like. In excipient-free conditions, the recombinant bromelain enzyme was affected by the rapid dehydration process and thermal change during spray drying. This condition occurs due to the liquid evaporation from the enzyme

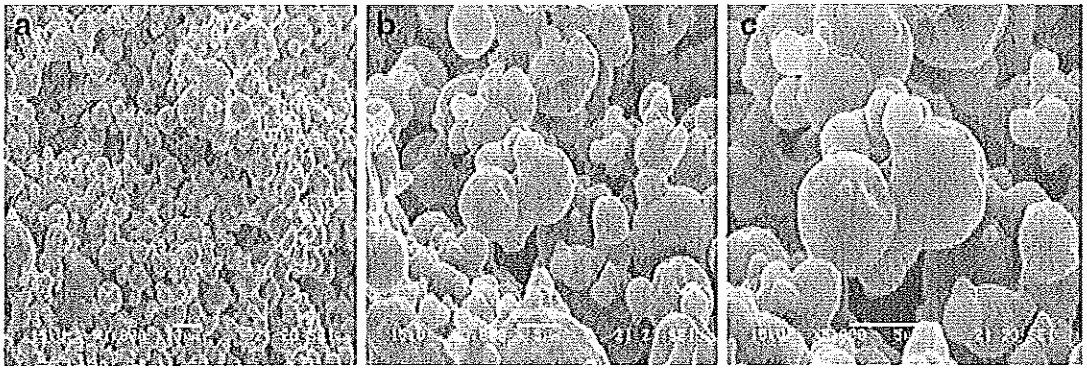

Fig. 12.12 SEM images of excipient-free spray dried recombinant bromelain. There stages of magnification at (*A*) 1000X, (*B*) 3000X and (*C*) 5000X

mixture and shrinkage of the droplets due to the water escaping during the transition of heat in the spray drying [13, 14]. The moisture content was relatively low due to the rapid water removal during the drying process, which reduced the surface area [14, 15].

It can be observed (negative control) with the naked eye that the excipient-free (2.23-fold) powder has a darker color and rough surface characteristics. Because of the low moisture content, the absorption of moisture from the ambient surroundings promotes the hygroscopicity of the dried powder.

In contrast with the recombinant bromelain mediated with maltodextrin as the excipient shown in Fig. 12.12, the powder had a smooth spherical shape, with a large particle size, and was non-porous and slightly damped. The function of excipients is not just preventing the recombinant enzyme solution from rapid dehydration but creating a cohesive film that maintains the heat associated with the rapidly hydrating sample [16]. By adding maltodextrin to the solution, the moisture content is relatively preserved, and thus, the slow activity loss of spray dried recombinant bromelain was prevented [17]. For other conditions, Phisut [17] mentioned that maltodextrin causes an increase in bulk density and inhibits dried powder hygroscopicity. In this study, the dextrose equivalent used was DE10. In addition, higher residual bromelain activity was achieved by applying moderate pump settings at 12% and maintaining the outlet temperature.

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