International Journal of Pharmaceutics, 76 (1991) 199–206

ADONIS 037851739100349R

IJP 02522

Preparation of proteolytic enzyme extracts from *Ananas comosus* L., Merr. fruit juice using semipermeable membrane, ammonium sulfate extraction, centrifugation and freeze-drying processes

M.B. Doko, V. Bassani *, J. Casadebaig, L. Cavailles and M. Jacob

Laboratoire de Pharmacie Galénique, Pharmacotechnie et Biopharmacie, Université de Montpellier I, Avenue Charles Flahault, 34060 Montpellier Cedex 01 (France)

(Received 19 March 1991)
(Accepted 26 April 1991)

Key words: *Ananas comosus* L., Merr.; Bromelain; Proteolytic activity; Microfiltration; Ultrafiltration; Diafiltration

Summary

Crude purified bromelain extracts were obtained from 0.26% protein pineapple juice using sequential batch membrane processing systems which included microfiltration (MF) and ultrafiltration (UF) followed by ammonium sulfate extraction, ultracentrifugation and freeze drying. The membrane treatments (with an 8 μm mineral MF and a 10000 molecular weight cut-off (MWCO) organic UF membranes), combined with 60% ammonium sulfate extraction resulted in 0.75–0.8% protein concentration, with 99% protein rejection. A 70% ammonium saturation and ultracentrifugation process (27000 $\times g$ at 2–3 °C), prior to freeze drying, were used in the last step to remove the residual non-protein constituents. These processes achieved low-moisture freeze-dried, and light-colored extracts, free of non-protein constituents and which accounted for about 50% yielded extracts containing 98% protein. The extracts assayed for bromelain and proteolytic activity resulted in almost 100% potential recovered, at completion. However, bromelain and proteolytic activity decay during the processes described above is essentially caused by losses through adsorption on the UF membrane relative to the level of concentration reached.

Introduction

Bromelain (EC 3.4.22.4) is a collective name for proteolytic enzymes or proteases found in tissues including stem, fruit, and leaves of the pineapple plant family Bromeliaceae. Isolation of the enzyme from pineapple fruit and its study have been investigated since 1894. The enzymes occurring in the stem and the fruit of *Ananas comosus* var. Smouth cayenne are the most studied (Ruyssen and Lauwers, 1978). Interest in bromelain, for its numerous applications in the food industry as well as in medicine and pharmacology, stems from its specific properties which make this enzyme one of the best vegetal proteases. The potential therapeutic value of bromelains, due to their biochemical and pharmacologi-

Correspondence: M.B. Doko, Laboratoire de Pharmacie Galénique, Pharmacotechnie et Biopharmacie, Université de Montpellier I, Ave Charles Flahault, 34060 Montpellier Cedex 01, France.

* On leave from the Faculty of Pharmacy of Porto Alegre, Federal University of Rio Grande do Sul, Brazil.

cal properties, has been reviewed (Dupaigne, 1975; Cooreman et al., 1976; Ruyssen and Lauwers, 1978; Taussig, 1980, 1984).

The preparation in pure form of a proteolytic enzyme has always proved difficult, and the bromelains appear to be no exception (Ota et al., 1964). Crude commercial bromelain used in the manufacture of pharmaceutical is not chemically homogeneous. Besides the main ingredient which is a proteolytic enzyme named glycoprotein, substances such as insoluble materials, e.g. colored pigments, organic acids, minerals, protease inhibitors, organic solvents and excipient used for enzyme recovery were detected (Murachi et al., 1964; Taussig, 1980). In addition, conventional methods used for the preparation of highly purified enzymes result in loss of stability and physiological activity. It is well known that ammonium sulfate is an effective complexing and extraction agent in protein isolation. Therefore, besides the simplicity and economy of the method, i.e. ammonium sulfate precipitation (Scopes, 1982), efficiency in the extraction and purification of enzymes has been demonstrated (Murachi et al., 1964; Tisseau, 1976; Aworh and Nakai, 1986; Guanthavorn and Powers, 1989).

The membrane technologies e.g. microfiltration (MF) and ultrafiltration (UF), as recent methods used to separate components of a solution based on molecular size differences (i.e., molecular sieving), are much required for liquid foods, in juice processing (Yu and Chiang, 1986; Yu et al., 1986; Rao et al., 1987; Sheu et al., 1987), as well as protein isolate and concentrate production (Olsen, 1978; Bérot et al., 1987; Deeslie and Cheryan, 1988; Tzeng et al., 1988), since besides reducing production costs, these processes show several other advantages for quality and production yield. Thus, the aims of this study were: (1) to evaluate the feasibility of membrane processing as a means of selective separation of protein from pineapple juice, and (2) to determine the efficiency of the combination of membrane processing, ammonium sulfate extraction, centrifugation and freeze-drying processes for the recovery of bromelain extracts with regard to quality, notably purity and enzymatic activity.

Materials and Methods

Pineapple juice preparation

The pineapple fruit (*Ananas comosus* L., Merr, Variety Smouth cayenne) used in the experiments is imported from the Ivory Coast. Preparation of the pineapple juice was conducted according to

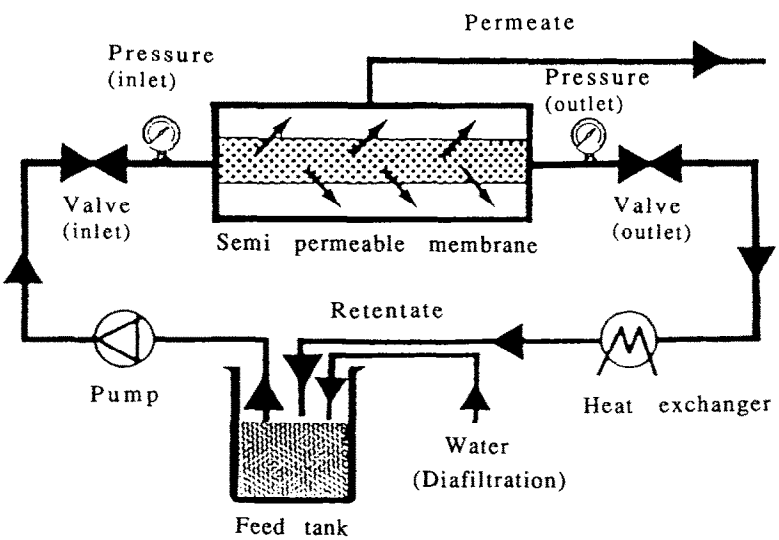

Fig. 1. Schematic diagram of batch membrane processing system for proteolytic enzyme recovery, from pineapple juice.

procedures described by Doko (1990). The resulting juice was analysed for protein (N × 6.25) and dry matter (DM), and stored at −25 °C for further experiments.

Membrane processing

The membrane processing, including MF and UF, was applied according to a batch processing system (Fig. 1). Since bromelain is heat sensitive

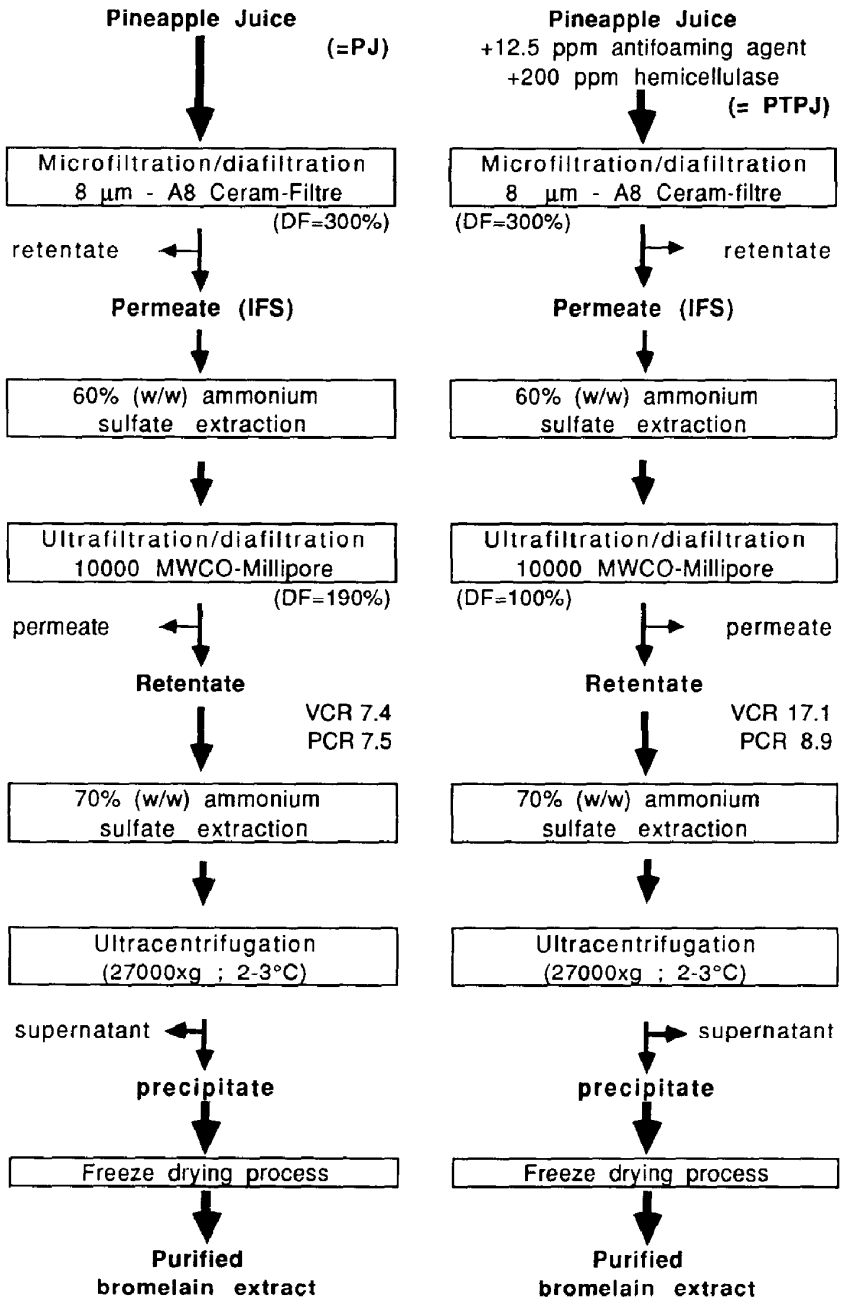

Fig. 2. Schematic flow diagram illustrating the steps involved in the purified bromelain extracts recoveries, from *Ananas comosus* L. Merr. fruit juices.

(Chen and Liu, 1972), the operating temperature in MF and UF processes was limited to a maximum of $30 \pm 2\,^{\circ}C$ by a heat exchanger. Diafiltration (DF) was carried out by addition of water in a feed solution tank at the rate of permeate removal. This was to facilitate, by both dilution and low molecular size draining, the MF and UF applications and thus, protein separation. DF was expressed as a percentage of the water on the basis of initial feed sample.

For MF, the equipment used was a pilot scale unit equipped with a volumetric pump type LCF4G (PCM-Pompes Moineau, Vanves, France) and an 8 μm mineral Ceram-filtre A8 membrane with a surface of 0.2 m^2 (Ceram-filtre, Lunel, France). This membrane is a tubular composite membrane made of zirconium oxide with carbure as support. Prior to the MF process, as indicated in Fig. 2, the sample of pineapple juice was submitted to pretreatments including enzyme treatment at pH 8.5 to hydrolyze pectic substances, thus reducing viscosity and improving filtration rate in membrane processing. Antifoam was used to prevent protein loss and to stabilize flow rate during membrane processing. The pH of pineapple juice samples was raised from 3.4 to 8.5 by addition of 30% aqueous NaOH solution. The enzyme treatment was conducted at $20\,^{\circ}C$ for 5 h using 200 ppm hemicellulose REG (Gist-Brocades Food Ingredients) with moderate stirring. Antifoaming treatment was carried out using 12.5 ppm Rhodorsil silicone oil HV 47350 (Rhône Poulenc, France).

For UF, a 10000 MWCO membrane was found to retain 100% proteins (e.g., the bromelain of which the molecular weight was about 30000). Before the UF processes, the saturation of the initial feed solutions (IFS) was carried out using 60% (w/w) of ammonium sulfate. The UF experiments were conducted with permeates recovered from MF (Fig. 2). The UF separation was processed, using an HPCF 230 F2 Millipore pilot plant equipped with an organic composite membrane (i.e., 10000 MWCO Millipore PTGC 000 05 cassette) made of polysulfone with polypropylene as support (Millipore Corp., Bedford, MA). This membrane (of area 0.46 m^2) can be used under specific conditions: pH 1–14 up to $50\,^{\circ}C$, operating pressure up to 7 bar. Inlet and outlet membrane operating pressures were 4 and 2 bar, respectively. During the UF run, after 5 min the permeates were collected separately, while retentates were returned to the feed tank, for concentration. Initial feed solutions (IFS), permeates and retentates were tested for crude protein (N $\times$ 6.25), and protein and volume concentration ratios (VCR, PCR). VCR is the initial batch volume of the feed divided by the final volume of the retentate, and PCR represents the retentate protein content divided by the initial feed protein content. The protein rejection is expressed as:

$$\%\text{Protein rejection} = (1 - C_p/C_r) \times 100$$

and protein recoveries relative to the protein concentration (C) in feeds (f), retentates (r) and permeates (p) and their corresponding volumes (V) are determined by the following equations:

$$\%\text{Protein yield in retentate} = (C_r V_r / C_f V_f) \times 100$$

$$\%\text{Protein yield in permeate} = (C_p V_p / C_f V_f) \times 100$$

(Omosaiye and Cheryan, 1979; Nichols and Cheryan, 1981), resulting (under optimal operating conditions, i.e., with overall fractions including retentate and permeate collected), in the solute mass balance equation expressed as:

$$C_f V_f = C_r V_r + C_p V_p + L_m$$

with L_m corresponding to solute loss (L) due to adsorption on the membrane m. The percentage of total solids, i.e. dry matter (DM), was determined from samples (4 ml) by drying in a $70\,^{\circ}C$ Chopin vacuum oven until constant weight. The degree protein purification expressed as a percent of protein on dry matter basis, was determined.

Ultracentrifugation (UC) and freeze drying (FD) processes

The retentates (20 ml) from the previous UF processes were 70% (w/w) saturated with ammonium sulfate and kept at $0\,^{\circ}C$ for 30 min, until UC at $27000 \times g$ for 30 min at $2–3\,^{\circ}C$ using a

Beckman JA20 rotor. The resulting precipitates were taken up in distilled water, and the washings were separated from the extracted slurry by a second step of a 15 min UC process. The purified protein isolates were recovered, using a Virtis Freeze dryer mobile 6 (Virtis Co. Inc., Gardner, NY, U.S.A.) freeze dryer under determined operating conditions (Doko, 1990). The resultant freeze-dried samples (FDS), after weighing, were analysed for percent residual water by drying samples (i.e. 2 g of FDS) at 105 °C to constant weight, production yields and purity as percentage of protein in the FDS.

The amounts of bromelain (in g/100 g of FDS) and the corresponding proteolytic activity expressed in terms of units of enzyme activity were determined from a standard curve using bromelain (EC 3.4.22.4, Sigma no. B-2252, lot 113F-0585; Sigma Chemical Co., St Louis, MO) of which the enzymatic activity was 1275 U/g of solid (Doko, 1990). This was determined according to the casein digestion method at 40 °C and pH 6 using 1% casein in the presence of cysteine and EDAT (AOAC, 1984). The assays were based on a 60 min proteolytic hydrolysis of the casein substrate. The unhydrolyzed substrate is precipitated with trichloroacetic acid and removed by filtration while solubilised casein (filtrate) absorbance is measured at 280 nm using a Shimadzu UV-visible 240 graphicord spectrophotometer.

Results and Discussion

For the recovery of crude bromelain extracts from pineapple juice samples, the extraction and purification processes schematically followed are set out as described in Fig. 2. Therefore, the steps of MF/DF served, besides dilution (at DF 300%), to remove excess pulp including fibers, pectic substances and other suspended solids in the pineapple juice, thus reducing dry matter (DM) to 4.3 and 3.7% from PJ and PTPJ of which initial DM values were 12.5 and 13.5, respectively (Table 1). Moreover, by significantly decreasing viscosity, the MF/DF processes provided suitable conditions to facilitate further UF membrane processing using a 10000 MWCO membrane. The protein contents in retentates from pineapple juice (PJ) and pretreated pineapple juice (PTPJ) were 0.75 and 0.80%, respectively. As expected, the protein rejection indicates that no protein permeated the membrane, i.e. protein retention was virtually 100%. Since a 10000 MWCO cassette was used in UF/DF steps, from PJ and PTPJ, the separation of proteins, mainly bromelain, having molecular weights (MW) above 10000 was thus completely controlled by the membrane-selective screening.

On the other hand, this separation process was detrimental to low-MW constituents, e.g. major soluble sugars including fructose, glucose and sucrose, organic acids, and minerals which were

TABLE 1

Membrane processing of pineapple juices, using an 8 μm membrane (for IFS) and a 10000 MWCO membrane for protein separation

Samples	%DF (water/IFS)	Dry matter	%Protein (N × 6.25)	%Protein rejection
Pineapple juice (= PJ)		12.5	0.26	
Initial feed solution (IFS)	300	4.3	0.10	
Permeate			0.01	
Retentate	190	2.3	0.75	99
Pretreated PJ (= PTPJ)		13.5	0.25	
Initial feed solution (IFS)	300	3.7	0.09	
Permeate			0.01	
Retentate	100	2.3	0.80	99

TABLE 2

Determination of protein separation efficiency, using 10 000 MWCO ultrafiltration / diafiltration processes from pineapple juice (PJ) and pretreated pineapple juice (PTPJ) samples

Samples	VCR	PCR	%Protein purification	%Protein recovery
Pineapple juice (= PJ)			2.1	
Initial feed solution (IFS)			2.3	
Permeate				
Retentate	7.4	7.5	32.6	97
Pretreated PJ (= PTPJ)			1.9	
Initial feed solution (IFS)			2.4	
Permeate				
Retentate	17.1	8.9	34.8	47

drained out, through the membrane. As a matter of fact, this transfer phenomenon, essentially improved by the DF process, reduced the concentration of permeable components, resulting in both a decrease of total solids (i.e. DM) in the retained protein solution and an increase of the protein/DM ratio. Therefore, the protein purification level in the retentates, expressed as percent of protein on DM basis increased, as shown in Table 2, from 2.3 to 32.6% and 2.4 to 34.8% from PJ and PTPJ, respectively. These findings indicated that even though pretreatments of pineapple juice resulted in a 17-fold higher VCR, compared with that obtained from PJ, i.e. 7.4, the protein content and protein purification level exhibited no further significant improvement than 0.80 and 34.8%, respectively. The best conditions, in the light of percent protein recoveries registered, are achieved with a value of 97%, at 7.4-fold VCR and 7.5-fold PCR, from PJ.

The following processes, i.e., 70% ammonium saturation, UC, used to remove the overall non-protein substances, resulted, after the FD process, in about 50% of protein in the retentates recovered as 2–3.5% moisture freeze-dried isolates containing 96.5–98% protein, as indicated in Table 3. Due to these processes, the removal of the overall non-protein residual substances (e.g., colored pigments) remaining in the retentates explains both the light-colored aspect, of the protein isolates, and the high 98 and 96.5% protein content of the resulting FDS, previously raised from an average of 2 to 32.6–34.8% protein purification (Table 2) by UF/DF applica-

TABLE 3

Bromelain and proteolytic activity recoveries from pineapple juices, using MP (with 8 μm and 10 000 MWCO membranes), ammonium extraction, UC and FD processes [a]

Samples	%FDS yield	%Residual water	%FDS protein	Bromelain (g/100 g FDS)	Total activity (Units)
Pineapple juice (= PJ)				0.45	574
Permeate				0.00	0
Retentate	47	2.0	98.0	1.27	1619
Pretreated PJ (= PTPJ)				0.60	765
Permeate				0.00	0
Retentate	50	3.5	96.5	1.21	1543

[a] The membrane process (MP), ammonium extraction, ultracentrifugation (UC) and freeze-drying processes were conducted as described in Fig. 2.

TABLE 4

Determination of bromelain separation efficiency, relative to concentrating factor (i.e. CF, determined from PJ and PTPJ), proteolytic activity ratio (PAR) using MP (with 8 μm and 10000 MWCO membranes), ammonium extraction, UC, and FD processes

Samples	%Protein (N×6.25)	CF	PAR	%Activity decay
Pineapple juice (= PJ)	0.26			
Permeate	0.01			
Retentate	0.75	2.9	2.8	3
Pretreated PJ (= PTPJ)	0.25			
Permeate	0.01			
Retentate	0.80	3.2	2.0	38

tions using a 10000 MWCO membrane, from PJ and PTPJ, respectively. When the crude extracts were assayed for bromelain recovery and proteolytic activity, the results in Table 3 show that, although enzyme treatment (PTPJ) provided the highest activity with 765 units, the greatest UF recovery was achieved without pretreatment, with 1.27% of bromelain on an FDS basis, and 1619 units as corresponding total proteolytic activity. The absolute bromelain retention of the 10000 MWCO cassette was confirmed by the absence of proteolytic activity in permeates, since of the permeate samples tested none showed a detectable level of bromelain.

Hence, in Table 4, comparison of the balance between concentration factor (CF) and proteolytic activity ratio (PAR) expressed on the basis of pineapple juice samples (i.e., PJ and PTPJ) revealed at 2.9-fold CF and 2.8-fold PAR no enzyme decay but 3% was observed on completion, from PJ, where optimun conditions were recorded, i.e., 97% protein recovered at 7.4-fold VCR and 7.5-fold PCR. This shows that, even though the pretreatments were designed to improve the efficiency of the UF process, beyond a value of about 7.5, the PCR did not increase linearly with VCR. Thus, as shown by the lowest 2-fold PAR (compared to the 3.2-fold CF), the decay of 38% bromelain activity consequently was due to protein losses by irreversible adsorption (i.e. immobilization) on the surface of the UF membrane and/or within its pores, since no enzyme activity was noted in permeates. The losses are due to increasing adsorption as the concentration level increases. Therefore, as the concentration processes increase with operational duration, enzyme recovery appears to decrease as solute exposure time to membrane processing increases. In addition, decay due to the physical destruction of bromelain (i.e., shear forces) or due to chemical destruction (i.e., enzyme self-destruction) are factors to be taken into account during membrane processing.

Conclusion

Membrane processing for the production of crude purified bromelain extracts appears to be quite effective. In addition, the diafiltration process, for greater solute separation, decreases total solids in the extracts, and permits a greater amount of subsequent UF processing, thus enabling better recovery of protein concentrates of high purity and concentration. The completion of bromelain production, by using a second step of ammonium saturation followed by ultracentrifugation and freeze-drying processes, yielded purified bromelain extracts, free of residual non-protein substances, and light in color. This, along with the advantages of improved results including yield, purity and enzyme activity preservation, makes these processes, i.e. the association of membrane processing, ammonium extraction, ultracentrifugation and freeze-drying, very attractive for the bromelain processing industry, since no organic solvents, inhibitor excipients or denaturation operation are involved.

Acknowledgments

The investigations were supported in part by ANVAR (Agence Nationale pour la Valorisation de la Recherche), within the contract ANVAR-UNIVERSITE no. A 86 06 075 J 073-0. This financial support is gratefully acknowledged. We are indebted to P. Legrand of Millipore Corp., France, for technical advice and for the generous supply of membranes for tests.

References

AOAC. Official methods of analysis, 14th Edn, Assoc. Offic. Anal. Chem. (1984), Arlington, VA.

Aworh, O.C. and Nakai, S., Extraction of milk clotting enzyme from sodom apple *(Calotropis procera). J. Food Sci.*, 51 (1986) 1569–1570.

Bérot, S., Guéguen, J. and Berthaud, C., Ultrafiltration of fababean (*Vicia faba* L.) protein extracts: process parameters and functional properties of the isolates. *Lebensm. Wiss. Technol.*, 20 (1987) 143–150.

Chen, Y.-M. and Liu, H.-Y., Studies on stem bromelain and stem starch from pineapple plants. *Taiwania*, 17 (1972) 266–276.

Cooreman, W.M., Scharpe, S., Demmeester, J. and Lauwers, A., Bromelain biochemical and pharmacological properties. *Pharm. Acta Helv.*, 51 (1976) 73–97.

Deeslie, W.D. and Cheryan, M., Functional properties of soy protein hydrolysates from a continuous ultrafiltration reactor. *J. Agric Food Chem.*, 36 (1988) 26–31.

Doko, M.B., Place de la filtration tangentielle dans les techniques séparatives pour l'optimisation de la production de l'enzyme protéolytique d'*Ananas comosus* L., Merr. Thesis (Doctorate) Montpellier I University, France (1990).

Dupaigne, P., Effets biochimiques des bromélines. Leur utilisation en thérapeutique. *Fruits*, 39 (1975) 545–560.

Fuh, W.-S. and Chiang B.-H., Purification of steviosides by membrane and ion exchange processes. *J. Food Sci.*, 55 (1990) 1454–1457.

Guanthavorn, C. and Powers, J.R., Partial purification and characterization of asparagus lipoxygenase. *J. Food Sci.*, 54 (1989) 371–373.

Murachi, T., Yasui, M., and Yasuda, Y., Purification and physical characterization of stem bromelain. *Biochemistry*, 3 (1964) 48–55.

Nichols, D.J. and Cheryan, M., Production of soy isolates by ultrafiltration: factors affecting yield and composition. *J. Food Sci.*, 46 (1981) 367–372.

Olsen, H.S., Continuous pilot plant production of bean protein by extraction, centrifugation, ultrafiltration and spray drying. *Lebensm. Wiss. Technol.*, 11 (1978) 57–64.

Omosaiye, O. and Cheryan, M., Ultrafiltration of soybean water extracts: processing characteristics and yields. *J. Food Sci.*, 44 (1979) 1027–1031.

Ota, S., Moore, S. and Stein, W.H., Preparation and chemical properties of purified stem and fruit bromelains. *Biochemistry*, 2 (1964) 180–185.

Rao, M.A., Acree, T.E., Cooley, H.J. and Ennis, R.W., Clarification of apple juice by hollow fiber ultrafiltration: fluxes and retention of odor-active volatiles. *J. Food Sci.*, 52 (1987) 375–377.

Ruyssen, R. and Lauwers, A., Bromelain (E.C. 3.4.22.4.). In Pharmaceutical Enzymes, Story-scientia PVBA Scientific Publ. Co., Gent/Belgium, 1978, pp. 107–121.

Scopes, R.L., *Protein Purification*, Springer-Verlag, New York, 1982.

Sheu, M.J., Wiley, R.C. and Schlimme, D.V., Solute and enzyme recoveries in apple clarification using ultrafiltration. *J. Food Sci.*, 52 (1987) 732–736.

Taussig, S.J., The mechanism of the physiological action of bromelain. *Med. Hypothesis*, 6 (1980) 99–104.

Taussig, S.J., Die Wirkung von Bromelain als Antikoagulans. *Erfahrungsheilkunde*, 33 (1984) 342.

Tisseau, R., Activité protéolytique de l'Ananas utilisé en conserverie et de ses déchets. *Fruits*, 31 (1976) 378.

Tzeng, Y.-M., Diosady, L.L. and Rubin L.J., Preparation of rapeseed protein isolate by sodium hexametaphosfate extraction, ultrafiltration, diafiltration, and ion-exchange. *J. Food Sci.*, 5 (1988) 1537–1541.

Yu, Z.R., Chiang, B.H., and Hwang, L.S., Retention of passion fruit juice compounds by ultrafiltration. *J. Food Sci.*, 51 (1986) 841.

Yu, Z.R. and Chiang, B.H., Passion fruit juice concentration by ultrafiltration and evaporation. *J. Food Sci.*, 6 (1986) 1501–1505.