Available online at www.elixirpublishers.com (Elixir International Journal)

Food Science

Elixir Food Science 45 (2012) 7822-7826

ISSN: 2229-712X

Isolation and characterization of Bromelain from pineapple (Ananas Comosus) and comparing its anti-browning activity on apple juice with commercial anti-browning agents

C.Ramalingam, R.Srinath* and N.Nasimun Islam

School of Biosciences and Technology, VIT University, Vellore-632001, Tamil Nadu, India.

ARTICLE INFO

Article history:

Received: 30 January 2012;

Received in revised form:

25 March 2012;

Accepted: 9 April 2012;

Keywords

Bromelain,

Ananas comosus,

Pineapple,

Protease and Anti browning agent.

ABSTRACT

Bromelain is a mixture of protein digesting (proteolytic) enzymes found in pineapples (Ananas comosus). The objective of this study was to assess the effectiveness of bromelain as an anti browning agent, extracted and purified from pineapple crown and pulp. The efficacy of extracted bromelain was compared with widely used anti browning agents. Bromelain purified by ion exchange chromatography was run through a SDS PAGE gel and was found to be having a molecular weight ranging from 20 to 30 kDa. The effect of pH and temperature on the protease activity of bromelain sample was also estimated. The anti browning activity was found out to be higher in the fruit pulp extract in comparison with the crown leaf extract as the former showed higher protease activity almost twice the crown leaf extract. Bromelain within the concentration range of 0.1% to 0.8% hardly proved to be a potent anti browning agent but 1% crown extract bromelain was a better anti browning agent than acetic acid and also fruit pulp bromelain was found to be a much better anti browning agent than ascorbic acid and acetic acid.

Introduction

Bromelain is a crude aqueous extract from the stems and immature fruits of pineapples, constituting an unusually complex mixture of different Thiol Endopeptidases and not yet completely characterized components such as phosphatases, glucosidases, peroxidases, cellulases, glycoproteins and carbohydrates. Bromelain can refer to one of two protease enzymes extracted from the plant family Bromeliacea. Bromelain extract is a mixture of protein-digesting enzymes called Proteases and several other substances in smaller quantity. The proteolytic enzymes are referred to as Sulfhydryl proteases since a free sulfhydryl group of a cysteine side-chain is required for the function. The two main enzymes are (a) Stem bromelain (EC 3.4.22.32) (b) Fruit bromelain (EC 3.4.22.33) The other substances typically include Peroxidase, Acid Phosohatase, Protease Inhibitor and Calcium. The stem bromelain is the most abundant cysteine endopeptidase in the pineapple's stem and exhibits a broad specificity for protein cleavage, having a strong preference for Z-Arg-Arg-I-NHMec amongst the small molecule substrates. The fruit bromelain is extracted from the pineapple's infrutescence. It hydrolyzes protein with a broad specificity for peptide bonds. Bz-Phe-Val-Arg-l-NHMec is a good synthetic substrate, but there is no action on Z-Arg-Arg-I-NHMec. Crude extract of bromelain mainly comprise glycosylated multiple enzyme species of the papain superfamily with different proteolytic activities, molecular masses between 20 and 31 kda, isoelectric point between >10 and 4.8. The enzyme activity comprises a wide spectrum with pH optimum between 5.5 and 8.0. The substrate spectrum is similarly broad, extending from synthetic low molecular mass amides and dipeptides up to high molecular mass substrate such as fibrin, albumin, casein, angiotensin ii, bradykinin. Bromelain preferentially cleaves glycyl, alanyl, leucyl bond and is absorbed through gastro-intestinal tract. It is widely used for therapeutic applications viz., (a) for oral systemic treatment of inflammation (b) prevention of platelet aggregation (c) as an effective fibrinolytic agent (d) modulation of immune cells function (by stimulating the production of tnf-α, il1-β and il6 in human peripheral blood mononuclear cells in a dose dependent manner (e) potentiation of antibodies (f) modulation of cell adhesion (g) inhibits tumor cell growth in vitro etc.

Enzymatic browning occurs in fruits and vegetables upon bruising during handling or transportation and when exposed to air in cut, sliced or pulped states (Labuza, T. and M.K. Schmidl, 1986). The initial enzymatic oxidation of phenols into slightly colored quinines can be defined as enzymatic browning (Nicolas et al, 1994). These quinines are subjected to further reactions which are enzymatically catalyzed leading to the formation of pigments (Ozoglu, H. and A. Bayindirli, 2002 and Wen, L. and R.E. Wrolstad, 2001). Polyphenol oxidases (PPO) have been associated with enzymatic browning as they are able to act on phenols in the presence of oxygen (Nicolas et al, 1994, Vamos-Vigyazo, 1981 and Sapers, G.M. and R.L. Miller, 1992). The quality, nutritional value and safety of foods are adversely affected as a result of enzymatic and non-enzymatic browning reactions (Molnar-Perl, I. and M. Friedman, 1990, Friedman, M., 1996, Laurila, E. and R. Ahvenainen, 2002, Tan, B.K. and N.D. Harris, 1995 and Billaud et al, 2003.) and washing with water is not effective in preventing the discoloration (Willey, R.C., 1994 and Mattila et al, 1995). Friedman (1996) identified reactions of amines, amino acids, peptides and proteins with reducing sugars and vitamin C (nonenzymatic browning) and quinones (enzymatic browning) to cause deterioration of foods during storage and commercial or domestic processing. The control of browning in order to maintain their quality, nutritional

Tele: +91- 9840975585

E-mail addresses: rsri2290@gmail.com

value and safety is of great importance just at the start of their processing procedures.

Anti-browning agents have been used for the prevention of enzymatic browning in fruit juices such as sulfites. However, sulfites use has been restricted due to health concerns (Anon, 1991). Laurila and Ahvenainen (2002) have noted that the most attractive way to inhibit browning would be by natural methods. Some natural agents that have been proposed to have inhibitory effect on PPO include honey (Oszmianski, J. and C.Y. Lee, 1990), natural aliphatic alcohols (Valero et al, 1990), and Millard Reaction Products (MRP) synthesized from glucose and lysine (Nicoli et al, 1991 and Pitotti et al, 1990). Only a few anti-browning agents have been considered as potential alternatives to sulfites although many inhibitors of PPO are known.

It was demonstrated that pineapple juice was an effective enzymatic browning inhibitor in fresh apple slices (Meza et al, 1995 and Lozano-de-Gonzales et al, 1993). The inhibitory effect of Stem Bromelain as anti-browning agent was also found to be very minimal (Tochi et al, 2009).

Thus this study aims to analyze the effectiveness of bromelain (fruit and crown leaf) as an anti-browning agent and concentration at which they are most effective. Also effect of other parameters such as temperature and pH are analyzed.

-MATERIALS AND METHODS-

Crude extraction of Bromelain

The crown leaves were thoroughly washed and dried. 50 grams of leaves were cut into small pieces. The leaves were homogenized in a blender by adding 75ml of pre-cooled sodium citrate buffer (Extraction buffer) and the stalk of ripe pineapple was thoroughly washed and dried. 50 grams of pulp were cut in to small pieces. The pulp was homogenized in a blender by adding 75ml of pre-cooled sodium citrate buffer (Extraction buffer). Both homogenates were filtered using a muslin cloth to remove fiber. The homogenate was centrifuged at 5000rpm for 15mins .The pellet obtained was discarded and the crude Protease was stored at 4^0C. Fresh red apples were purchased from local market and were washed and peeled to remove the outer layer. Apples were crushed using the kitchen blender; the resulting juice was filtered using muslin cloth. The juice was transferred into new eppendorf tubes containing anti-browning agents. Only apple juice was taken as a control. Samples were incubated at room temperature for 1hr and reading for each sample was taken at 420nm for every 10mins. The percentage of inhibition was calculated using values coming from control and fractions.

Inhibition % = A (420 nm) control – A (420 nm) treatment * 100 / A (420 nm) control.

Ammonium sulfate precipitation

The precipitation of bromelain was carried using 50 ml of the juice extract for both stem and pulp individually. Ammonium sulfate was added to get the required saturation from the saturation table under constant stirring using a magnetic stirrer.

The stirring was continued for 10mins after complete addition of the ammonium sulfate to allow attainment of equilibrium between the dissolved and aggregated protein. The salt enriched solution was then subjected to centrifugation at 5000rpm for 15mins and the precipitate was collected. The supernatant was recollected and the volume was utilized for further saturation with ammonium sulfate.

Dialysis

The dialysis membrane is filled with bromelain juice obtained from ammonium sulfate fractionation and sealed. The dialysis membrane is kept in a beaker containing pre-cooled sodium citrate buffer. The dialysis bag along with the buffer is stirred using magnetic stirrer in a cold room. The buffer was changed for every 1hr.

Ion Exchange Chromatography

The CM cellulose resin was prepared in acetate buffer pH 4. The dialyzed soup was mixed with equilibrated CM cellulose and incubated for 1 hr at room temperature with intermittent mixing. The CM cellulose with the dialyzed soup was allowed to flow through a mini column having beads inside for controlling the flow rate.

The matrix was allowed to settle down by the gravity and collect the fluid which contains the unbound protein in an eppendorf tube. Cellulose matrix is then covered with a porous filter paper. The matrix is eluted (gradient elution method) by increasing the salt concentration in elution buffer.

Molecular Weight Determination by SDS-PAGE

The molecular weight of the purified protein was determined under denaturing conditions by subjecting the sample to 12% SDS-PAGE. The protein sample was run along with standard molecular weight marker and bands were visualized using Silver staining technique.

Protease assay: Azo-casein assay

50µl of 1% azo-casein was added to 30µl of fractionate and incubated for 1hr at room temperature. To this solution, 120µl of 10% TCA was added. The resulting pellet was precipitated by centrifugation. To 70µl of supernatant, 160µl of 1N NaOH was added. The absorbance of yellow color was observed at 440nm and values recorded.

Protein estimation method: Lowry's method

Different concentration of BSA ranges, 0.2mg/ml to 1 mg/ml were prepared and followed standard Lowry's method by measuring optical density at 660nm. The protein concentration of the unknown sample was estimated using the calibrated standard graph.

Determination of Optimum pH

The effect of pH on the protease activity of bromelain sample was estimated by using azo-casein as substrate. The activity of azo-casein substrate was measured at various pH ranges, by using phosphate, acetate and citrate buffers.

Determination of Thermal Stability

The effect of temperature on protease activity was evaluated by azo-casein assay. The bromelain sample was subjected to various temperatures from 40° to 80°C for 15 minutes**.** After incubation, the samples were cooled in ice for 5mins, brought to room temperature and determined for their residual protease activity.

-RESULTS-

OD at 660nm for crude leaf extract and fruit pulp extract is 0.492 and 0.517 respectively. Total volume of crude leaf extract and fruit pulp extract was 75ml.

Concentration of crude leaf and fruit pulp extract was found out to be 0.824 mg/ml and 0.872 mg/ml respectively. The specific activity of leaf extract and pulp was found out to be 3.83unit/mg and 9.82units/mg respectively.

Total amount of protein = (concentration) x (total volume of extract).

Table 1: Observation table for Lowry's method

BSA (ml)	WATER (ml)	SAMPLE CONC. (mg/ml)	ALKALINE COPPER SULFATE (ml)	LOWRY'S REAGENT (ml)		OD At 660nm
0.2	0.8	0.2	1	0.2		0.148
0.4	0.6	0.4	1	0.2	Incubate	0.273
0.6	0.4	0.6	1	0.2	for 30 min	0.375
0.8	0.2	0.8	1	0.2	In dark	0.485
1.0	0	1.0	1	0.2		0.620
Crude leaf extract 1ml	0	0.824	1	0.2		0.492
Crude pulp extract 1ml	0	0.872	1	0.2		0.517
Blank	1.0	-	1	0.2		0.00

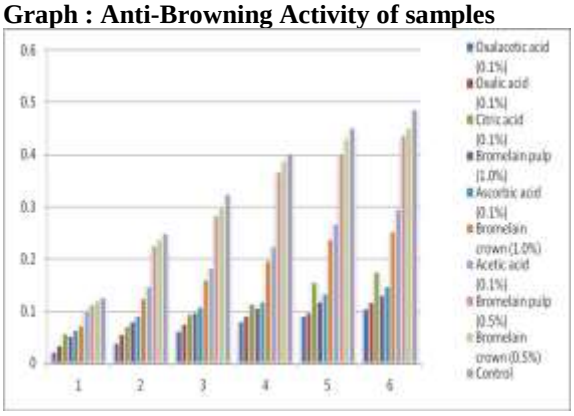
Table 2: Total Protein Content and Specific activity

TOTAL VOLUME	OD at 660nm	CONCENTRATION (mg/ml)	TOTAL AMOUNT OF PROTEIN (mg)	Total protease activity (units)	Specific activity (units/mg)
Leaf extract: 75ml	0.492	0.824	61.8	236.8	3.83
Pulp extract: 75ml	0.517	0.872	65.4	642.4	9.82

Table 3: Comparison of Anti-Browning Activity of fractionated samples with commercial agents

Anti-browning agents	OD Value (420nm) at an interval of 10 min					
	10 min	20 min	30 min	40 min	50 min	60 min
Bromelain pulp (0.1%)	0.113	0.227	0.285	0.367	0.403	0.437
Bromelain pulp (1.0%)	0.053	0.081	0.098	0.107	0.119	0.132
Bromelain crown (0.1%)	0.121	0.238	0.302	0.389	0.431	0.452
Bromelain crown (1.0%)	0.073	0.125	0.161	0.197	0.239	0.254
Acetic acid (0.1%)	0.103	0.148	0.184	0.225	0.268	0.296
Oxalic acid (0.1%)	0.035	0.057	0.076	0.091	0.098	0.117
Oxaloacetic acid (0.1%)	0.023	0.039	0.062	0.081	0.092	0.106
Citric acid (0.1%)	0.058	0.072	0.094	0.115	0.156	0.176
Ascorbic acid (0.1%)	0.064	0.092	0.109	0.119	0.135	0.148
Control	0.127	0.249	0.325	0.403	0.452	0.487

Graph : Anti-Browning Activity of samples



X-axis = Time interval of 10mins

Y-axis = O.D. value of Apple juice and Browning inhibitor at 420nm

Determination of molecular weight by SDS PAGE

The molecular weight of CM cellulose purified bromelain was determined by 12% SDS PAGE. The fractions collected from CM cellulose showed more than one band in SDS PAGE indicating that bromelain was not completely purified but two bands were observed between 20 kDa and 30 kDa, which might correspond to bromelain bands

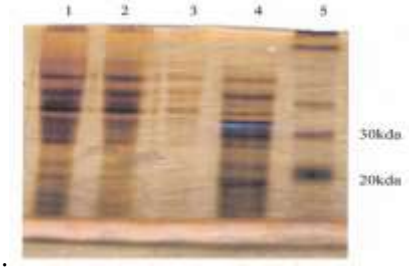


Figure - SDS PAGE showing protease bands after passing through CM cellulose resin: Lane 1: Crude sample, Lane 2: Flow through, Lane 3: Wash, Lane 4: Elution and Lane 5: Pre stained protein marker

Determination of Optimal pH

The effect of pH on the protease activity of the bromelain was studied in the range of 4-8 by using the azo-casein assay. The bromelain from crown leaf showed maximum activity at pH 6, where as the fruit pulp showed maximum activity at pH 8.

Determination of Thermal Stability

The optimal temperature was determined for both crown leaf and fruit pulp purified bromelain samples. Both the samples

were active when incubated at 50ºC for 15 minutes. At higher temperatures, the enzyme was gradually inactivated. The complete inactivation was observed for crown leaf at 70ºC and the fruit pulp bromelain showed complete inactivation at 80ºC.

-DISCUSSION-

Crude bromelain is a mixture of a cystein proteases that catalyses the hydrolytic cleavage of the internal peptide bonds of the protein substrate. Bromelain was isolated and purified from crown leaf and fruit pineapple. The purification process was achieved by ammonium sulfate fractionation, followed by dialysis and then cation exchange chromatography. The protease activity of purified bromelain was determined by azo-casein assay. The anti-browning activity of the crude bromelain was also determined.

In Ammonium Sulfate precipitation, the fractionation was carried out in three different ranges: 0-20%, 20-40%, 40-70% and above 70%. Each fraction was assayed for its protease activity by Azo-casein assay.

In case of crown leaf extract, only 20-40% fraction showed protease activity while other fractions did not show any activity indicating that the entire protein was fractionated in a single fraction. In contrary, protease activity was observed in both 20-40% and 40-70% fractions in case of fruit pulp extract. To further characterize, 40-70% saturation interval was divided into smaller fractions: 40-60% and 60-70% and the protease activity was checked with these fractions. Only 40-60% fractions showed some activity, while 60-70% did not, indicating that by 60% saturation level, most of the protease was precipitated out of the solution.

Bromelain obtained using ion exchange chromatography was run through SDS PAGE and was found to be having a molecular weight ranging from 20 to 30 kDa. The protein content was found to be higher in the fruit pulp extract and corresponding protease activity was found out to be approximately twice the protease activity of the crown leaf extract. The anti-browning activity was found out to be higher in the fruit pulp extract in comparison with the crown leaf extract as the former showed higher protease activity almost twice the crown leaf extract. This increase has been highly correlated with the protein content as the fruit pulp extract contained higher protein content they showed higher protease activity and in turn higher anti-browning activity. Various other parameters were analyzed like pH and temperature activity, crown leaf protease showed maximum activity at pH 6 and was inactivated at 70ºC, whereas fruit bromelain showed maximum activity at pH 8 and was completely inactive at 80ºC.

The study showed that bromelain is a weak anti-browning agent when compared with some of the available commercial anti-browning agents. All of the commercially available anti-browning agents were taken at a concentration of 0.1gm/100 ml (0.1%). Amongst the anti-browning agents taken for study Oxaloacetic acid is the most effective followed by oxalic, citric acid, ascorbic acid, acetic acid and least was bromelain. The bromelain extracted from the pineapple crown was less effective in preventing browning than the bromelain extracted from the fruit pulp. This can be explained by the fact that protein content in the fruit pulp was found out to be more than the crown part which directly correlates it to the amount of protease present (i.e.) amount of protease is more in the fruit pulp extract. Bromelain within the concentration range of 0.1% to 0.8% hardly proved to be a potent anti-browning agent but with the concentration of 1% bromelain crown proved to be a better anti-browning agent than acetic acid whereas, bromelain extracted from the fruit pulp proved to be a better anti-browning agent than ascorbic acid and acetic acid with a concentration of 1%.

Although commercial anti-browning agents are widely used, some are being restricted and banned by FAO such as sulfites. Even with many beneficial effects there are several negative attributes associated with sulfite use which has led to decreased consumer acceptance. In particular, sulfites can induce severe allergic reactions or even anaphylactic shock in a proportion of the asthmatic population. Hence there is a need for natural anti-browning agents which can be used in the food industry.

-CONCLUSION-

As the study was aimed at understanding the effectiveness of bromelain as a potential anti-browning agent we utilized the commercially available anti-browning agents for comparison. As observed fruit bromelain at a concentration of 1% has the potential to be a better agent than the widely used ascorbic acid and but its potential use in long term replacement for the chemically synthesized anti-browning agents is still premature. Although it provides a basis for the use of natural alternatives in the food industry, its use is difficult due to the required concentration and purification techniques involved thereby increasing the cost of preparing such anti browning agents. Hence a better option would be to use those easily and cheaply available commercial agents which have not been found to have any hazardous or deleterious effects on the food.

However further studies about in-vitro gene amplification for increasing bromelain yield, improved purification and concentration techniques for bromelain can help identify and better analyze the potency and use of bromelain as a natural anti-browning agent in the food industry.

-REFERENCES-

- Anon, 1991. Sulphites banned. Food Ingredients Process. Int., 11: 11-11.
- B.N. Tochi, Z. Wang, Shi Ying Xu and W. Zhang, 2009. Effect of Stem Bromelain on the Browning of Apple Juice. American Journal of Food Technology, 4: 146-153.
- Billaud, C., E. Roux, Brun-Me′rimee, S. Maraschin and C.J. Nicolas, 2003. Inhibitory effect of unheated and heated d-glucose, d-fructose and l-cysteine solutions and Millard reaction product model systems on polyphenol oxidase from apple. I. Enzymatic browning and enzyme activity inhibition using Spectrophotometric and polarographic methods. Food Chem., 81: 35-50.
- Friedman, M., 1996. Food browning and its prevention: An overview. J. Agric. Food Chem., 44: 631-653.
- Friedman, M., 1996. Food browning and its prevention: An overview. J. Agric. Food Chem., 44: 631-653.
- Labuza, T. and M.K. Schmidl, 1986. Advances in the Control of Browning Reactions in Foods. In: Role of Chemistry in the Quality of Processed Food, Fennema, O.R., W. Chang and C. Lii (Eds.). Food and Nutrition Press Inc., Westport, pp: 65-94.
- Laurila, E. and R. Ahvenainen, 2002. Minimal Processing of Fresh Fruits and Vegetables. In: Fruits and Vegetable Processing-Improving Quality, Wim, J. (Ed.). CRC Press, Washington, DC., pp: 1-22.
- Lozano-de-Gonzales, P.G., D.M. Barrett, R.E. Wrolstad and R.W. Durst, 1993. Enzymatic browning inhibited in fresh and dried apple rings by pineapple juice. J. Food Sci., 58: 399-404.
- Mattila, M., R. Ahvenainen and E. Hurme, 1995. Prevention of browning of pre-peeled Potato. Proceedings of Workshop on Systems and Operations for Post-Harvest Quality, COST 94

Post-Harvest Treatment of Fruit and Vegetables, 1995, Belgium, pp: 225-234.

- Meza, J., P. Lozano-de-Gonzales, A. Anzaldua-Morales, J.V. Torres and J. Jimenes, 1995. Addition of pineapple juice for the prevention of discoloration and textural changes of apple slices. Institute of Food Technologists Annual Meeting, Book of Abstracts.
- Molnar-Perl, I. and M. Friedman, 1990. Inhibition of browning by sulphur Amino Acids. 2. Fruit juices and protein containing foods. J. Agric. Food Chem., 38: 1648-1651.
- Nicolas, J.J., F.C. Richard-Forget, P.M. Goupy, M.J. Amiot and S.Y. Aubert, 1994. Enzymatic browning reactions in apple and products. Crit. Rev. Food Sci. Nutr., 34: 109-157.
- Nicoli, M.C., B.E. Elizalde, A. Pitotti and C.R. Lerici, 1991. Effect of sugar and Millard reaction products on polyphenol oxidase and peroxidase activity in food. J. Food Biochem., 15: 169-184.
- Oszmianski, J. and C.Y. Lee, 1990. Inhibition of polyphenol oxidase activity and browning by honey. J. Agric. Food Chem., 38: 1892-1895.
- Ozoglu, H. and A. Bayindirli, 2002. Inhibition of enzymic browning in cloudy apple juice with selected antibrowning agents. Food Control, 13: 213-221.
- Pitotti, A., C. Nicoli, A. Sensidon and C.R. Lerici, 1990. Control of Enzymatic Browning in Food: Effects of Some Substances and their Mechanism Action in Engineering and Food. In: Physical Properties and Process Control, Spiess, W.E.L. and H. Schuberts (Eds.). Elsevier Sciences Publisher, London, UK.
- Sapers, G.M. and R.L. Miller, 1992. Enzymatic browning control in potato with ascorbic acid-2-phosphates. J. Food Sci., 57: 1132-1135.
- Tan, B.K. and N.D. Harris, 1995. Millard reaction products inhibit apple Polyphenoloxidase. Food Chem., 53: 267-273.
- Valero, E., R. Varon and F. Gracia-Carmona, 1990. Inhibition of grape polyphenol oxidase by several aliphatic alcohols. J. Agric. Food Chem., 38: 1097-1100.
- Vamos-Vigyazo, L., 1981. Polyphenol oxidase and peroxidase in fruits and vegetables. Crit. Rev. Food Sci., 15: 49-127.
- Wen, L. and R.E. Wrolstad, 2001. Pineapple juice: Phenolic composition and enzymatic browning inhibition. Ph.D. Thesis, Oregon State University.
- Willey, R.C., 1994. Minimally Processed Refrigerated Fruits and Vegetables. Chapman and Hall, New York.