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Purification and characterization of Limonoate-D-ring lactone hydrolase from kinnow fruits grown in Punjab, India

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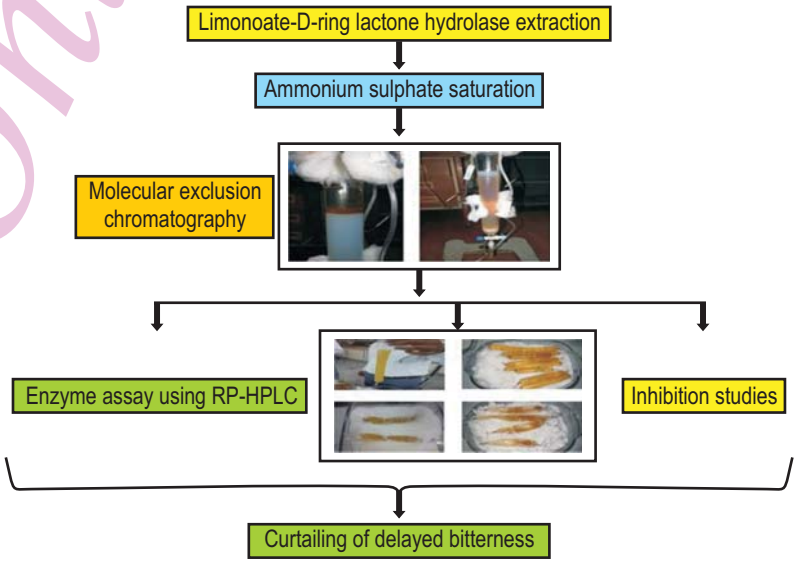
Abstract

Aim: The enzyme limonoate-D-ring lactone hydrolase (LDLH) is known to cause delayed bitterness in kinnow fruit juice. This enzyme catalyzes the conversion of non-bitter precursor, limonoate-A-ring lactone/LARL to bitter limonin in acidic medium of juice.

Methodology: Limonoate-D-ring lactone hydrolase was extracted from kinnow seeds and purified using ammonium sulphate fractionation (25-85%) followed by molecular exclusion chromatography (seralose-CL-6B). Enzyme activity was inhibited with food grade inhibitors and enzyme assay was carried out with high pressure liquid chromatography.

Results: This procedure resulted in 74.57 fold purification and recovery of 5.02%. Molecular mass of the purified enzyme was found to be 224 kDa, while subunit molecular mass of 45 kDa was adjudged using gel electrophoresis suggesting its pentameric nature. Bromelain treatment inactivated the enzyme by 11.5%; sodium hexmetaphosphate, L-glutamate and 1,2-cyclohexylenedinitrilotetraacetic acid could inhibit enzyme activity by ~40%; while ethylenediamine tetraacetic acid caused 58.1% inhibition. Papain, pepsin, histidine and DL-malic acid did not have any discernable effect on its activity.

Interpretation: Sodium hexmetaphosphate, L-glutamate, 1,2-cyclohexylene dinitrilotetraacetic acid and EDTA can be used for inhibiting LDLH activity in kinnow juice to curtail limonin generation, thereby reducing delayed bitterness.



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Introduction

Delayed bitterness is the major problem in kinnow juice processing that has low market demand and consumer acceptability (Puri *et al.*, 2002). In fact, the US public health services in 1969 classified bitterness in citrus juices as one of the most interesting and complex problems in food science and technology (Premi *et al.*, 1994; Siddiqui *et al.*, 2013). Limonin is the primary component of limonoid metabolism responsible for causing bitterness in citrus juices (Fayoux *et al.*, 2007). It is a bitter, white, crystalline substance and often found at higher concentrations in citrus fruit seeds (Yoon *et al.*, 2010). An intact citrus fruit hardly contains limonin but when the juice is extracted, the acidic pH of the juice facilitates the conversion of LARL (a non-bitter precursor) to limonin (bitter) leading to the development of delayed bitterness in citrus juice (Hasegawa and Maier, 1990) and this reaction is catalyzed by the action of an enzyme, limonoate D-ring lactone hydrolase (LDLH). The enzyme LDLH, catalyzing the reversible conversion of LARL into limonin was first isolated from citrus seed by Maier *et al.* (1969). The limonin precursor (LARL) is naturally confined to membranous juice sacs of citrus fruits and once the juice sacs gets ruptured during juice extraction, the enzyme LDLH becomes activated that gradually converts LARL into limonin which imparts delayed bitterness to kinnow juice (Ferreira *et al.*, 2008; Maier *et al.,* 1969). The bitterness in citrus juice is also caused by naringin (another bittering compound) but limonin contributes to more intense bitterness compared to the naringin (Puri *et al.*, 1996). The limonoid aglycones are responsible for the development of delayed bitterness in citrus and get converted to non-bitter limonoid glucosides during fruit maturation (Hasegawa *et al.*, 1989). The delayed bitterness in kinnow juice can be prevented if the juices are made alkaline (Khandelwal *et al.,* 2006) or by the process that irreversibly converts LARL into its dehydro form, and thus avoiding the production of limonin (Hasegawa and Maier, 1990).

Kinnow mandarin (*Citrus nobilis x C. deliciosa)* is one of the important citrus fruit crops of northern India. The annual production of mandarins (including oranges and kinnows) was about 3.43 million metric tones with an area of cultivation of about 330 thousand hectares for year 2013-14 (Indian Horticulture Database, 2015)**.** Its fruits are mainly used for juice purpose but naturally occurring limonin (a limonoid) and naringin (a flavonoid) are the de-facto bitterness causing chemical constituents that adversely affect consumer acceptability of juice and allied products (Ferreira *et al.,* 2008; Hasegawa *et al.,* 1989; Khandelwal *et al.,* 2006; Puri *et al.,* 2005). Limonoid bitterness gradually occurs after juice processing from kinnow fruit which is referred to as delayed bitterness and the reaction is catalyzed by LDLH enzyme by formation of limonin. Due to this bitterness, 95% of kinnow fruits are used for table purpose and only 5% are processed into juice or juice concentrate **(**Khandelwal *et al.,* 2006) rendering market glut and price fall during peak production season. As a result, both growers and processors suffer huge loss in the absence of desired post-harvest technology, required infrastructure and availability of cost effective technology for de-bittering of kinnow juices. Though considerable research effort has been made in the past for detecting and modulating limonoids in relationship to the development of "delayed bitterness" in kinnow but a low cost process has not been developed. Also, accumulation of limonoids in seeds is complicated and entirely different from other fruit tissue, and thus purification and characterization of LDLH enzyme from kinnow seed has not been studied extensively. In addition, this enzyme can eventually be used to develop biosensors for the determination of limonin levels in juices. Hence, the present study aimed to purify, characterize and work out regulatory properties of LDLH enzyme in order to produce sweetened juices using food grade inhibitors for making kinnow juice processing industry commercially viable.

Materials and Methods

Raw material : Healthy and disease free kinnow fruits were obtained at full maturity from local orchards at Abohar region, Punjab, India. The fruits were immediately brought to Post Harvest Technology Laboratory of Horticultural Crop Processing Division, washed with 100 ppm chlorinated water and the juice was extracted using screw press. The seeds were separated from fruits after juice extraction, washed and dried under shade at room temperature. The dried seeds were packed in LDPE bags and stored under ambient conditions until their intended use. All chemicals and lab accessories used during the investigations were of analytical grade.

Preparation of crude extract : The enzyme LDLH was extracted by the method adopted from Breska III and Manners (2004) with slight modification (omitting the use of sodium dodecyl sulphate). The crude extract was prepared by homogenizing 1.5 kg kinnow seeds with 0.1 M Tris extraction buffer (pH 8.0) containing 3% PVP and 1% NaCl. The resulting broth was placed in a shaker (150 rpm) for 3 hrs and then filtered through double layer of cheesecloth. The extract so obtained was centrifuged in a refrigerated centrifuge (Eltak, India) at 7000 rpm for 60 min and the supernatant was filtered using bloating paper and grade-4 filter paper. The enzyme was further sequentially purified by ammonium sulphate fractionation (25-85% saturation) and molecular exclusion chromatography using seralose CL-6B.

Purification of LDLH : The process comprised of preparation of crude extract, salt saturation by ammonium sulphate and molecular exclusion chromatography using seralose CL-6B. The crude extract was subjected to precipitation with ammonium sulphate. Preliminary standardization was carried out for optimizing enzyme yield during ammonium sulphate saturation and based on that, the crude enzyme was subjected to 0-25% $(NH_4)_2SO_4$ saturation for 5 hrs and centrifuged at 7,000 rpm for 40 min. The supernatant was

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collected and the precipitate was discarded as it had negligible LDLH activity. The resulting supernatant was again brought to 85% $(NH_4)_2SO_4$ saturation. The precipitate collected after 5 hrs by centrifugation (7000 rpm, 40 min) was analyzed for LDLH activity while the supernatant was subjected to 100% $(NH_4)_2SO_4$ saturation for 5 hrs and centrifuged at 7,000 rpm for 40 min. The resulting pellet (85-100% saturation) was also analyzed for LDLH activity. The 25-85% fraction having enough LDLH activity was re-dissolved in minimum amount of Tris buffer (0.1 M; pH 8.0); the volume was reduced osmotically and dialyzed for 24 hrs against the same buffer with repeated changes of buffer. The dialyzed fraction was concentrated by osmosis against solid sucrose and loaded onto a Seralose CL-6B (1.8 x 22 cm) column pre-equilibrated with 0.1 M Tris buffer (pH 8.0) for 10 hrs. The enzyme was eluted with same buffer at a flow rate of 25 ml hr^{-1}. The fractions of 5.0 ml each were collected for protein and LDLH estimation. The protein was analyzed spectrophotometrically at 280 nm (Shimadzu, Japan), while LDLH activity was determined at 210 nm using reverse phase high pressure liquid chromatography (RP-HPLC). The active fractions showing maximum LDLH activity were concentrated using sucrose (osmosis) and used as purified LDLH enzyme. The concentration of protein at each step was determined by the method of Bradford (1976), using bovine serum albumin (BSA) as standard.

Enzyme assay of LDLH : Since LARL was not available commercially, so the activity of LDLH was quantified in reversible manner at alkaline pH using limonin as substrate. The crude/purified LDLH was filtered through 0.45 µm nylon membrane and used for HPLC analysis. The HPLC (Model: D-2000 Elite LaChrome; Make: Hitachi, Japan) used in the present investigation consisted of reverse phase C18 column and diode array detector (DAD). Enzyme LDLH was assayed using method of Breska III and Manners (2004) with slight modifications. For LDLH enzyme, 520 µl of Tris buffer (0.1 M; pH 8.0), 50 µl of 40% acetonitrile, 400 µl of 2 mM limonin and appropriately diluted enzyme extract were mixed thoroughly and incubated at 37°C in a water bath for a pre-determined time. After completion of reaction, 20 µl of 0.25 M EDTA was added to the reaction mixture. A control containing all the above ingredients except enzyme was also run simultaneously and 80 µl each of control and reacted mixture was fed to HPLC for analysis. The decrease in limonin concentration was monitored at 210 nm with a flow rate of 1 ml min^{-1} through DAD. The peak area was quantified and converted to ppm using limonin standard curve and expressed in ppm of limonin degraded/min.

Characterization of LDLH

Determination of molecular mass : Molecular mass of purified enzyme was determined by molecular exclusion chromatography on a Seralose CL-6B column. The column was calibrated for molecular mass with standard molecular mass markers (kDa); bovine serum albumin (66.0), lactate dehydrogenase (140.0), catalase (232.0), apoferritin (440.0) and bovine thyroglobulin (669.0). The molecular mass of enzyme was determined using a plot of elution volume *vs.* log molecular masses of standard proteins. A regression equation y = -0.0019x + 2.9857 was computed statistically and used to know molecular mass of purified LDLH.

Poly acrylamide gel electrophoresis (PAGE) : Native PAGE was performed as per the procedure of Davis (1964). The samples were loaded onto 4% stacking gel at an electric current of 20 mA while the bands were resolved on resolving gel (6%) at 40 mA. The standard molecular mass markers (kDa) used for native PAGE were bovine serum albumin (66.0), lactate dehydrogenase (140.0), catalase (232.0), apoferritin (440.0) and bovine thyroglobulin (669.0). Sodium do-decyl sulphate polyacrylamide (disc) gel electrophoresis (SDS-PAGE) was performed as per the procedure of Laemmili (1970). The samples were loaded onto 4% stacking gel with a flowing electric current of 20 mA while the bands were resolved on resolving gel (10%) at current of 30 mA. The standard molecular mass markers (kDa) used were bovine serum albumin (66.0), ovalbumin (45.0), pepsin (36.0), carbonic anhydrase (29.0) and lysozyme (14.3). The gels were stained with Coomassie Brilliant Blue R250 and then de-stained the excess dye. The gels were then photographed for further analysis.

Inhibitor studies on LDLH : The purified LDLH enzyme was treated with 50 units each of papain, bromelain and pepsin and the broth was incubated for 1 h at 50°C in a water bath. Rest of the inhibitors, *viz*. EDTA, histidine, 1,2-cyclohexylenedinitrilotetra-acetic acid (CDTA), L-glutamate, sodium hexmetaphosphate and DL-malic acid were added directly to the reaction mixture @ 20 µl of 20 mM concentration and inhibition of LDLH activity was determined in each case by using HPLC. The purified enzyme was heated at 95°C for 5, 10 and 15 min in a water bath to assess the heat stability and effect of pasteurization temperature on enzyme inactivation.

Results and Discussion

The LDLH enzyme catalyzes reversible conversion of LARL to limonin depending upon pH. Under acidic conditions of citrus juices, the LARL precursor (non-bitter) is converted to limonin (bitter), causing delayed bitterness. At least 53 different limonoids have been isolated from citrus and its closely related genera but four limonoids namely, limonin, nomilin, ichangin and nomolinic acid are bitter in taste (Bayazit and Konar, 2010). Limonin is the primary component of limonoid metabolism responsible for causing bitterness in citrus juices (Siddiqui *et al*., 2013). The LDLH enzyme was extracted from kinnow seed and purified using a two stepped process that involved ammonium sulphate precipitation and molecular exclusion chromatography. Following each step, total protein and total activity decreased, whereas specific activity increased (Table 1). Specific activity of LDLH was found to be higher (19.67 units mg^{-1} protein) in 25-85%

Table 1 : Summary of purification of LDLH of kinnow seeds

Purification step	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units mg^{-1} protein)	Fold purification	Yield (%)
Crude extract	7800.0	60440.6± 28.39	458640.0±28.31	7.59	1.00	100.0
Ammonium sulphate saturation (25-85%)	1026.0	4485.7± 16.46	88236.0± 21.98	19.67	2.59	19.24
Molecular exclusion chromatography (Seralose CL-6B)	13.0	40.7± 0.98	23037.2± 18.10	566.02	74.57	5.02

Values are mean of three replicates ± SEm

Table 2 : Relative inhibition (%) of purified LDLH activity by various inhibitors

Inhibitor	Concentration	Relative inhibition (%)
Papain	50 U	N.D.
Bromelain	50 U	11.50±0.46
Pepsin	50 U	N.D.
EDTA	20 mM	58.13±0.58
Histidine	20 mM	N.D.
CDTA	20 mM	41.42±1.53
L-glutamate	20 mM	40.60±1.29
Sodium hexmetaphosphate	20 mM	40.10±0.91
DL-malic acid	20 mM	N.D.

N.D. = Not detected (any inhibition); Values are mean of three replicates ± SEm

ammonium sulphate saturated fraction compared to 0-25 and 85-100% fraction. The fold purification and overall yield of this fraction were 2.59 fold and 19.24%, respectively. Thus, 25-85% fraction was selected for further processing. The total volume (1026.0 ml) of this fraction was reduced via osmosis using solid sucrose; dialyzed against 0.1 M Tris buffer (pH 8.0) using semipermeable membrane for 24 hrs with slow and constant stirring with repeated changes of buffer. The sample was concentrated to 60 ml by osmosis and loaded onto seralose CL-6B column for molecular exclusion chromatography. Fractions with LDLH activity (fraction number 68-82) were pooled and used for further characterization (Fig. 1). The specific activity of purified LDLH was found to be 566.02 units mg^{-1} protein. Molecular exclusion chromatography resulted in 74.57 fold purification of LDLH with 5.02% yield (Table 1). The enzyme reaction depends on pH of reaction mixture. Limonin producing lactonization of D-ring of LARL takes place at pH 6.0, while its reverse (*i.e.* hydrolysis of limonin D-ring to yield LARL) happens at pH 8.0 (Schomburg and Salzmann, 2013). Very few reports of purification and characterization of LDLH enzyme are available in literature. Merino *et al.* (1996) purified LDLH from citrus seeds using ammonium sulphate precipitation and ion exchange chromatography on DEAE-sepharose to 744 fold with 23% yield. A limonin degrading enzyme, limonin dehydrogenase from periplasmic fraction of *Pseudomonas putida* G7 was purified to 26 fold with an overall yield of 26% using gel filtration, hydroxyapatite, anion exchange and monoQ chromatographies (Malik *et al.*, 2012). Limonoate dehydrogenase was purified from cell free extracts of *Aspergillus globiformis* using ammonium sulphate fractionation, Cibacron blue affinity chromatography and DEAE ion exchange HPLC to a fold purification of 428 folds and with a molecular weight of 31 kDa (Teranishi *et al.*, 2012). Molecular mass as determined by gel filtration was found to be 224 kDa. This is in agreement to that deduced by Merino *et al.* (1996).

In order to test the purity of purified LDLH, native PAGE was performed using standard molecular mass markers. The native PAGE of purified LDLH showed a single band indicating the purity of LDLH (Fig. 2). However, the molecular mass as determined on the basis of native-PAGE was 250 kDa. Subunit molecular mass of purified enzyme as judged by SDS-PAGE was found to be approximately 45 kDa, thus indicating its pentameric nature with five equal subunits (Fig. 3). Merino *et al.* (1996) reported the subunit molecular mass of citrus LDLH between 43-45 kDa. Malik *et al.* (2012) reported that limonin dehydrogenase purified of *Pseudomonas putida* G7 was a monomer and had molecular weight of 26 kDa.

The LDLH enzyme characterization was aimed at working out its possible inactivation and/or inhibiting the conversion of non-bitter LARL into bitter limonin under acidic conditions that occurs during juice extraction due to rupturing of

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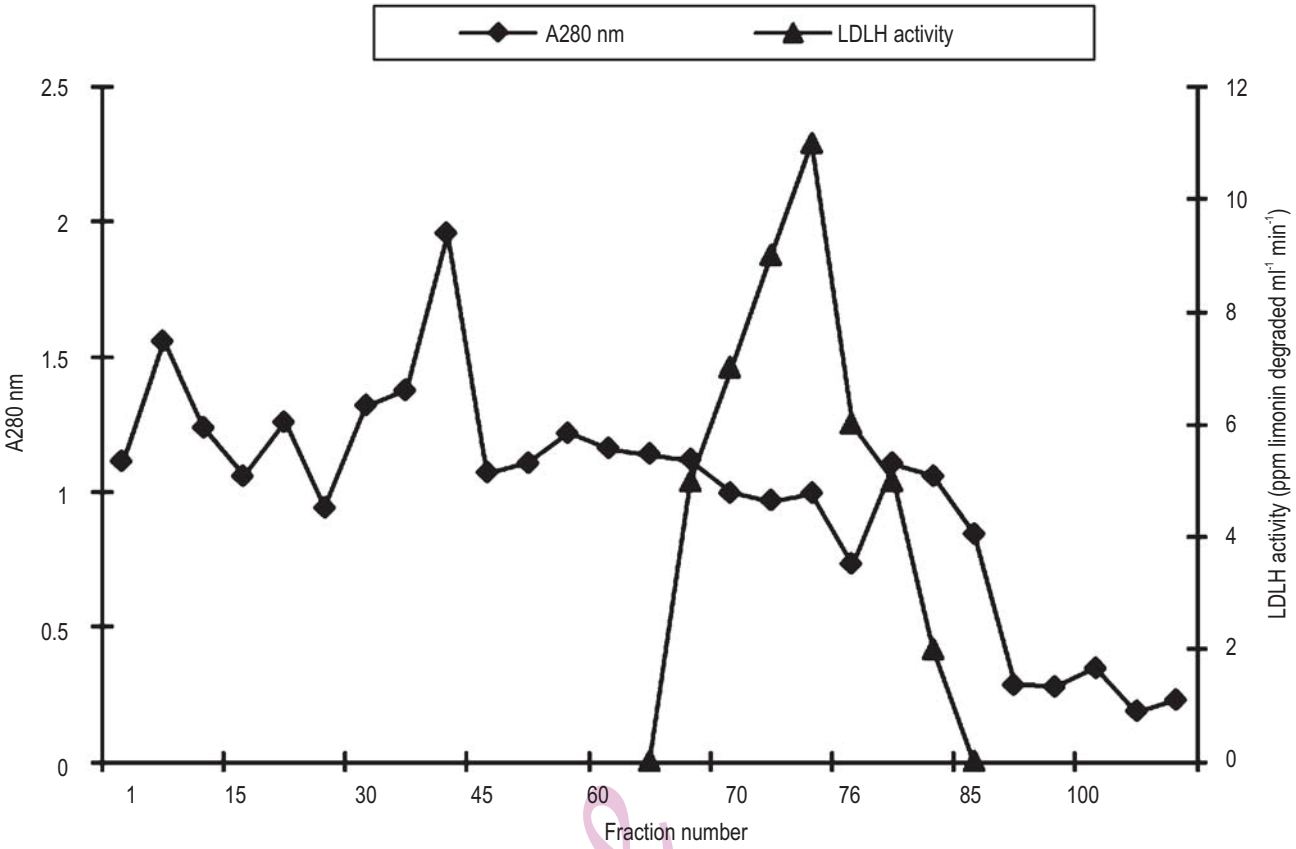
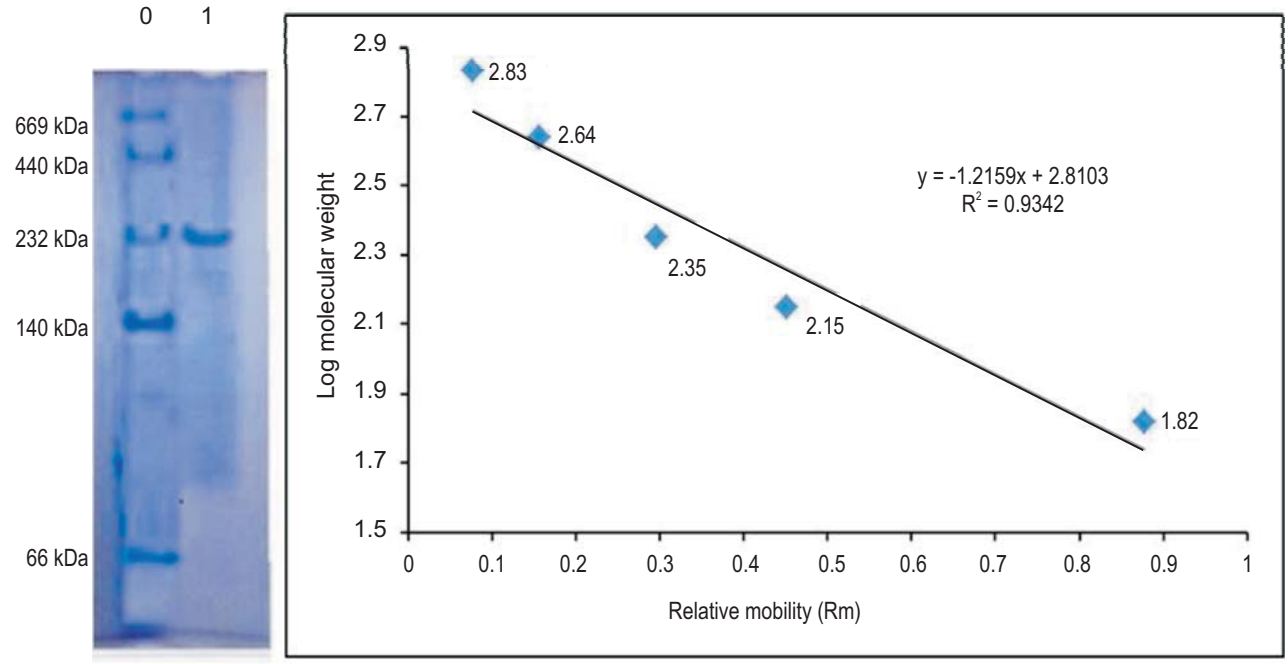


Fig. 1 : Elution profile of LDLH on Seralose CL-6B column

juice sacs. Several methods have been used to circumvent limonin formation or to eliminate limonoid from citrus juices so as to produce the acceptable quality of juice (Kola *et al.*, 2009; Malik *et al.*, 2012). Limonoate dehydrogenase (LDase), an enzyme detected in different species of microorganisms can prevent limonin production by catalyzing the irreversible oxidation of LARL to the corresponding 17-dehydrolimonoate form, a non-bitter derivative which cannot be converted into limonin (Malik *et al.*, 2012a**)**. However, the optimal pH of this enzyme (8-9) hampered the use of isolated enzyme in de-bittering processes due to the acidic pH of juices (Teranishi *et al.*, 2012). Thus, in the current investigation, we tried to inhibit LDLH activity at the de-facto pH of juice. The LDLH inactivation/inhibition was carried out using papain, pepsin and bromelain, the non-specific proteases and non-enzymic inhibitors, listed in Table 2 and heat treatment studies. Of the three proteolytic enzymes, pepsin and papain did not affect LDLH activity to any extent, whereas bromelain inactivated the enzyme by 11.5%, which might be due to pH optima of pepsin, bromelain and papain which correspond to 2, 4 and 6, respectively. As pH of experimental kinnow juice was near 4.0; only bromelain could inhibit enzyme activity in extracted juices to some extent, while others could not. Therefore, an immobilized cell system might be an option to protect protelolytic enzymes from external pH for improved (LDLH) inhibition/degradation by other proteolytic enzymes (Teranishi *et al.*, 2012).

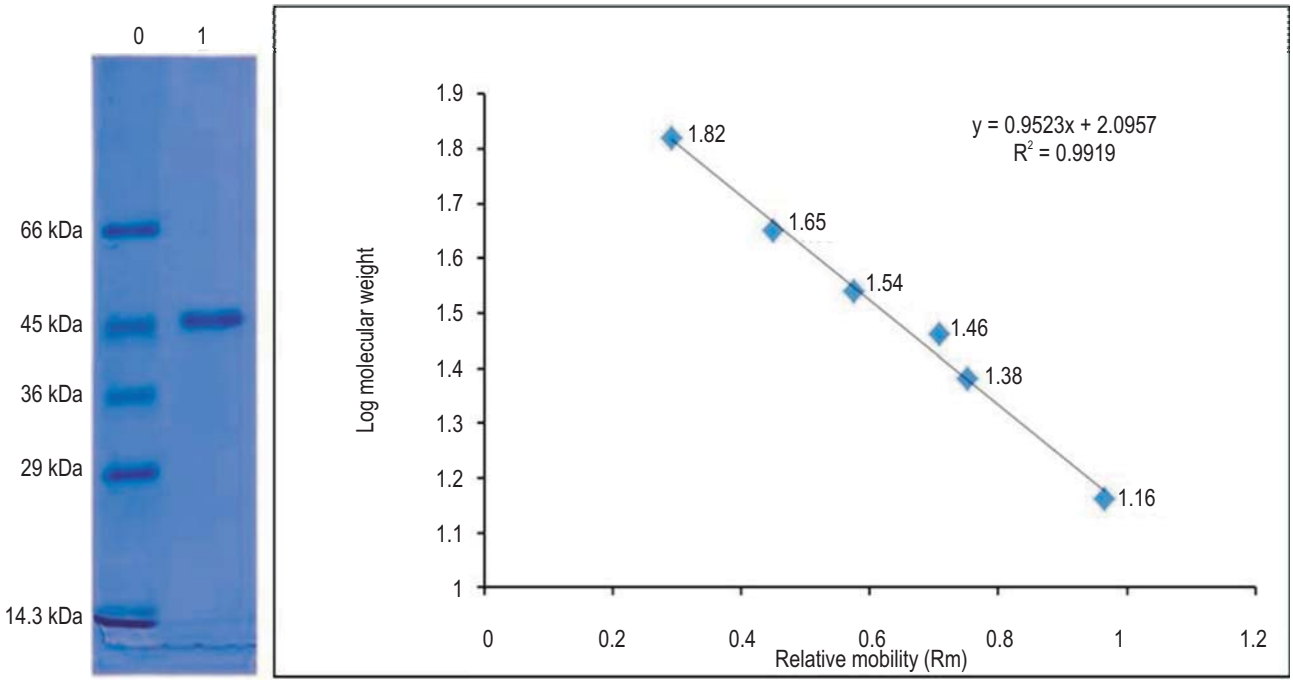
The data presented in Table 2 further revealed that histidine and DL-malic acid failed to show any effect while sodium hexmetaphosphate, L-glutamate and CDTA inhibited enzymatic activity by ~40%. EDTA alone was found to be the most potent one, as it inhibited enzyme activity by 58.1%. Hence, EDTA, CDTA, L-glutamate and sodium hexmetaphosphate may be used to inhibit LDLH mediated conversion of LARL to limonin in kinnow juice (Table 2). Thirdly, heating the enzyme preparation at 95°C for 15 min resulted in 15% loss of enzyme activity, while 5 and 10 min heating had no effect. These results are in conformity with Merino *et al.* (1996), where heating the crude enzyme extract to 95°C for 5 min had no effect on its activity.

It may be concluded from the results that LDLH could be purified to 74.57 fold with 5.02% yield using ammonium sulphate precipitation and molecular exclusion chromatography using seralose CL-6B. Sodium hexmetaphosphate, L-glutamate, 1,2-cyclohexylene dinitrilotetraacetic acid and EDTA can be used for inhibiting LDLH activity in kinnow juice to curtail limonin generation, thereby reducing delayed bitterness.



Lane 0: Standard markers
Lane 1: Purified enzyme

Fig. 2 : Native PAGE profile of purified limonoate-D-ring lactone hydrolase



Lane 0 : Standard markers
Lane 1 : LDLH subunit band

Fig. 3 : SDS-PAGE profile of purified limonoate-D-ring lactone hydrolase

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