Biotechnological synthesis, immobilization, characterization and comparison of novel anti-viral drug as Protease Inhibitor from Bacillus subtilis-M15

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Abstract

Protease Inhibitors belong to class of drug which are used to cure or prevent infection by viruses like HIV and HCV. Protease Inhibitors from Bacillus subtilis-M15 prevent viral multiplication by inhibiting the activity of Trypsin and Pepsin. Pharmaceutically designed ion-exchange resins are proved as promising candidates to enhance the Inhibitory activities of Protease. The novel Biological anti-viral drugs are characterized by Hydrogen Peroxide, Dimethyl Sulfur, HCl, and Acidic-Alkaline Proteinase. When Protease Inhibitory activity of chemically formulated drug already available in market i.e. Lopinavir/Ritonavir are compared with novel anti-viral drug from Bacillus subtilis-M15 then Lopinavir/Ritonavir are proved as less effective than Biological drug due to their lower Inhibitory activity in terms of units per milliliter. Lopinavir/Ritonavir has 2446U/ml and 241U/ml Protease Inhibitory activities against trypsin and pepsin respectively whereas Biological drug from Bacillus subtilis-M15 has 2581U/ml and 3360U/ml Protease Inhibitory activities against Trypsin and Pepsin respectively. In this context Biological drug developing treatment has attracted much attention than chemically designed drug due to their high efficacy, least side effect, cost efficient and environment friendly nature. Hence we conclude the current application of novel anti-viral drug as a Protease Inhibitors from Bacillus subtilis-M15 and its future clinical prospects against targeted Protease based HIV and HCV therapy.

Introduction

The agents, which can lower the catalytic activity of enzymes, are known as enzyme inhibitors. Enzyme inhibitors work as therapeutic agents and regulators to control enzyme activities in living organisms. These also behave as a useful tool to study the structure and mechanism of action of enzyme[1–3]. The vital function of Protease Inhibitor (PIs) is to control the unnecessary proteolysis and have an important function in physiological regulation. The efficiency of Protease Inhibitors depends upon the protease that they inhibit. To understand PIs, it is necessary to understand protease regulation and its proteolytic activity. The proteolytic activities of proteases are monitored and need to be under control. The Association of an enzyme with their respective inhibitors is an effective regulatory mechanism. Definite inhibition of proteases with their respective inhibitor is a special technique adopted for drug designing. PIs are used as a diagnostic tool against different bacterial, parasitic, fungal, and viral diseases in medicine. PIs may also play their role as therapeutic agents for treating cancer, cardiovascular and immunological diseases[4].

Approximately 50,000 natural products are obtained from the microorganism. Among them, 10,000 have been reported for biological activity, and more than 100 microbial products are used nowadays as anti-tumor agents and antibiotics. PIs from microbial sources are identified and reported in several applications[5]. To identify a novel compound, there is a need to isolate novel microbial PIs from natural sources like bacteria, fungi, and microbe[6]. Protease inhibitors (PIs) are proteins naturally occurring in living organisms and able to inhibit and control the activity of proteases. They are ubiquitous proteins occurring in animals, microorganisms, and plants[7]. PIs are primarily classified based on the class of

protease that they inhibit; thus, four main inhibitor families have been established: serine protease inhibitors, cysteine protease inhibitors, metalloprotease inhibitors, and aspartyl protease inhibitors[8]. Different protease inhibitors inactivate the target protease in human diseases like high blood pressure, arthritis, pancreatitis, thrombosis, muscular dystrophy, cancer, and AIDS. For the last ten years, scientists and pharmacists have tried to make drugs from protease inhibitors due to their mechanism of action against killing of disease causing viruses like malaria, HIV, cancer, influenza, and Alzheimer[9]. Protease inhibitors can block protease activity and convert proteins into peptides. Pepstatin has the potential ability to degrade aspartic protease[10].

For the treatment of acquired immunodeficiency syndrome (AIDS), the US Food and Drug Administration has approved 25 compounds for the clinical uses which are included around 10 Protease Inhibitors, i.e., ritonavir, fosamprenavir, saquinavir, indinavir, atazanavir, amprenavir, nelfinavir, darunavir, lopinavir and tipranavir[11]. It has been found from various studies that the Natural products played a vital role in the prevention and treatment of various diseases in humans. The quality of natural products may be evaluated through (i) introduction rate for a new chemical entity with wide structural diversities, which can serve as a template for synthetic as well as semisynthetic modifications[12]. (ii) total number of the diseases which can be prevented or treated through using these natural products[13] (iii) the natural product use frequency for treatment of human diseases.

Along with the progress in computer-based drug design in microbiology, there is a strong need to combat diseases like Human Immunodeficiency Virus (HIV), Alzheimer's disease and cancer[14–16]. Microbial enzymes are the preferred source of industrial enzymes as they are produced in large quantities in a short period and have shorter generation times. Genetic manipulations are performed more easily on bacterial cells to increase enzyme production. Pepstatin A (HIV-1 Protease Inhibitor) is a potent reversible inhibitor of aspartic proteases like pepsin and HIV proteases. Pepstatin A inhibits pepsin at picomolar concentrations. Statine, an amino acid, is thought to be responsible for the inhibitory activity of pepstatin A. That is because it mimics the tetrahedral transition state of the peptide catalysis[17].

Methodology

Sample collection for bacterial strains

Bacterial strains were isolated from different soil samples of Government College University, Lahore, Pakistan Botanic Garden, Tolinton Market Lahore, and a water sample from a hot spring in Azad Kashmir. Soil solution (1%) was prepared for serial dilution. It have been confirmed that the experimental samples of bacteria, including the collection of bacteria, complied with relevant institutional, national, and international guidelines and legislation with appropriate permissions from authorities of the Government College University, Lahore, Pakistan Botanic Garden; Tolinton Market Lahore, and from authorities of ‘Azad Kashmir’ Pakistan. Soil solution with suitable dilution (10^{-5}-10^{-6}) was transferred

aseptically on prepared Petri plates. Prepared Petri plates were placed in a 37^{o} centigrade incubator for 48 h selected strains were transferred to prepared slants and kept in the incubator at 37 °C.

Preparation of fermenting media for an anti-viral drug

For the synthesis of a novel anti-viral drug from *Bacillus subtilis*-M15, the following sub-merged biotechnological method of fermentation was adopted. 2 g of soybean meal, 1.5 g of glucose, and 2 g of peptone, 0.1 g $(NH_4)_2SO_4$, 0.1 g KH_2PO_4, and 0.5 g sodium carbonate was weighed and mixed in 70 ml of distilled water. Water was mixed continuously until marked with 100 ml of that solution. Then it was autoclaved for 15 min. for 15 lb/inch^2 pressure at 121 °C. Then 0.5 ml of inoculum was mixed in fermentation media. The flask was placed in a shaker for 48 h at 37°C with a 200 rev/min revolution speed. Then after 48 h, the sample was centrifuged with 6000 revolutions per minute for 10 min. Supernatant and protein precipitation was kept in a safe place.

Assay for Protease Inhibitory Activity of the anti-viral drug

Protease Inhibitory activity of the anti-viral drug was calculated with few amendments by Kunitz's (1947)[18] methods. 1 ml of Trypsin/Pepsin was mixed with 1 ml of Protease Inhibitor for 15 min with an optimum temperature of 37°C. Then 2ml of casein (1%) was transferred to the test as mentioned above tube for 30min. 2.5ml 0f TCA (0.44M) was added to the test tube for the reaction termination. In this method, the TCA soluble fractions were formed by the action of trypsin/pepsin on the protein substrate. Hammerstein casein was measured by the change in absorbance at 280 nm. The residual caseinolytic activity of the trypsin/pepsin in the presence of an inhibitor at 37 °C was used to measure inhibitory activity. Appropriate blanks for enzyme inhibitors and substrate were included in the assay. The mixture was further processed for 15 min in a centrifugation machine with a speed of 1000rpm. Then absorbance of the supernatant was taken at 280nm. The highest protease inhibitory activity of the anti-viral drug depicted the high efficacy and good potency of a drug against viral protease.

Partial purification of the anti-viral drug (Biological PI)

Ammonium sulphate $(NH_4)_2SO_4$ was added with the enzyme at a temperature of 4°C. The continuous addition of $(NH_4)_2SO_4$ was confirmed and marked up to 70% of solution saturation. Then this solution was placed at 4 °C for 10 min. After 30 min, the test tube sample was centrifuged at 10000 revolutions for 10 min. The precipitated enzyme (pellet) was dissolved in a minimum amount of 0.1 M Tris-HCl buffer solution, and then it was dialyzed. Then 10 ml of dissolved pellets were added to a given dialysis tube of length 10 cm and width of 25 mm and kept in 1000 ml of 0.1 M Tris-HCl buffer solution with continuous stirring for 24 h at 4°C buffer was refreshed 3-4 times during this period[19].

The immobilizing technique (Physical adsorption)

5 ml of partially purified protease inhibitor was taken, and 0.5 g of each adsorbent denoted as S1 to S7 will be continuously stirred and placed in the water bath at 37° centigrade with the speed of 100 rpm for

60 min. Then it was centrifuged for 10 min with 6000 revs. Then the supernatant was used to calculate the activity of protease inhibitor[20].

Characterization of Antiviral Drug

Effect of oxidizing agent on Protease Inhibitory activity of anti-viral drug

The impact of oxidizing agents was measured through incubation of anti-viral drug (biological protease inhibitor) with 1, 2, 3, 4, and 5% (v/v) of hydrogen peroxide and dimethyl sulfoxide (DMSO) for 30 min. Protease Inhibitory Activity was measured as mentioned before.

Effect different supports on Protease Inhibitory activity of anti-viral drug

Different immobilizing supports/ion-exchange resins like Amberlite, Doulite, Lewatit, Dowex 66, Dowex 80, IR 401 and IR100 were used to enhance the Protease Inhibitory activity anti-viral drug. Different ion exchanges and resins were checked using the immobilizing technique mentioned above. Protease Inhibitory Activity was measured.

Effect of Acid Treatment on Protease Inhibitory activity of anti-viral drug

The sensitivity of anti-viral drugs in an acidic environment was evaluated by incubating purified protease inhibitors with different concentrations of HCl ranging from 0.02, 0.04, 0.06, 0.08 and 0.I M (pH 2.0) for 30 min. After the incubation, pH was neutralized with 1 m1 of 0. l M Tris-HCl buffer pH 9.0. Protease Inhibitory Activity was estimated.

Effect of Protease Treatment on Protease Inhibitory activity of anti-viral drug

The sensitivity of anti-viral drugs to gastric enzymes like trypsin/pepsin was assessed by incubating the partially purified protease inhibitor with different concentrations of trypsin/pepsin ranging from 0.2, 0.4, 0.6, 0.8, and 1% for 30 minutes at 37 °C. Protease Inhibitory Activity was estimated.

Comparison of Protease Inhibitory activity of biological anti-viral drug and ritonavir (synthetic drug)

Protease Inhibitory Activity of Ritonavir (synthetic Protease Inhibitor of HIV-AIDS, Cancer and HCV) was calculated through standard assay technique as described above for anti-viral drug as Protease Inhibitor. The value of the Inhibitory Activity of Ritonavir was compared with the Inhibitory Activity of a novel anti-viral drug.

Results

Inhibitory activity of 25 bacterial proteases was determined before and after partial purification. After partial purification, the inhibitory activity of bacterial protease became enhanced. All the 25 bacterial different strains values were denoted from M1 to M25. Among 25 bacterial strains, two have shown maximum protease inhibitory activity, 14.2U/ml for bacterial strain M15 (*Bacillus subtilis*) and 16.1U/ml

for bacterial strain M24 in crude form. After partial purification, the enhanced values of bacterial strain were 89U/ml for M15 (*Bacillus subtilis*) and 97U/ml for bacterial M24 (Fig. 1)

Seven different ion exchange resins (from S1 to S7) were used for immobilization. Lewisite, Dowex 80, Dowex 66, Duolite, Amberlite, 1R 100, and IR401 were denoted with S1, S2, S3, S4, S5, S6 and S7, respectively (Table 1). Protease inhibitory activity of bacterial strain M15 (*Bacillus subtilis*) against trypsin (Alkaline Protease) was calculated through immobilization. Immobilized PIs showed enhanced activity compared to the partially purified sample of the same bacterial strain. Dowex 80 showed the highest value (13) fold of purification than Lewatite, which showed the least (9.2) purification fold. An immobilized sample of PIs against pepsin (Acidic Protease) showed enhanced activity compared to a partially purified sample of the same bacterial strain. IR 401 showed the highest value (13.3) fold of purification than Lewatite, which showed the least (9.4) purification fold.

Table 1
Protease Inhibitory activity of *Bacillus subtilis*-M15against pepsin and trypsin with ion exchange resins

SI = Lewatite	**S3 = Dowex 66**	**S5 = Amberlite**	**S7 = IR401**	
S2 = Dowex 80	**S4 = Doulite**	**S6 = IR100**		
Ion Exchange Resins	**Protease Inhibitory activity with Trypsin (U/ml)**	**Folds of Immobilization**	**Protease Inhibitory activity with Pepsin (U/ml)**	**Folds of Immobilization**
S1	132	9.2	210	9.4
S2	167	13	270	12.2
S3	152	10.8	253	11.5
S4	165	11.6	268	12
S5	172	12.1	292	13
S6	157	11	281	12.6
S7	138	9.7	298	13.3

Effects of hydrogen peroxide on protease inhibitory activity were calculated for bacterial strain M15 against trypsin (Alkaline Protease) and pepsin (Acidic Protease) through standard assay as mentioned earlier. Protease inhibitory activity was checked for five concentrations of Hydrogen peroxide (V/V %). Bacterial strain M15 showed the highest (15.3) Inhibitory Activity (U/ml) at 4% (v/v) Hydrogen peroxide concentration against trypsin. Bacterial strain M15 showed highest (23.4) inhibitory activity (U/ml) at 3% (v/v) Hydrogen peroxide concentration against pepsin (Table 2).

Table 2
Protease Inhibitory activity of `Bacillus subtilis-M15 against Pepsin and Trypsin with Hydrogen peroxide

Hydrogen Peroxide V/V (%)	Protease Inhibitory Activity with Trypsin (U/ml)	Protease Inhibitory Activity with Pepsin (U/ml)
1	14.3	20.8
2	13.2	21.3
3	12.1	23.4
4	15.3	22.7
5	13.7	20.6

Effects of dimethyl sulfoxide on protease inhibitory activity were calculated for bacterial strain M15 against trypsin (Alkaline Protease) and pepsin (Acidic Protease) through standard assay as mentioned earlier. Protease inhibitory activity was checked for five different concentrations of dimethyl sulfoxide (V/V %). Bacterial strain M15 showed highest (17.3) Inhibitory activity (U/ml) at 2% (v/v) dimethyl sulfoxide concentration against trypsin (Table 3). Bacterial strain M15 showed the highest (23) Inhibitory activity (U/ml) at 4% (v/v) dimethyl sulfoxide concentration against pepsin.

Table 3
Protease Inhibitory activity of `*Bacillus subtilis*-M15 against Pepsin and Trypsin with Dimethyl sulfoxide (DMSO)

Dimethyl Sulfoxide (DMSO) V/V (%)	Protease Inhibitory Activity with Trypsin (U/ml)	Protease Inhibitory Activity with Pepsin (U/ml)
1	14	21.5
2	17.3	20.9
3	11.2	22.5
4	15	23
5	13.2	19.8

Effects of HCl (Molar concentration) on protease inhibitory activity was calculated for bacterial strain M15 against trypsin (Alkaline Protease) and pepsin (Acidic Protease) through standard assay as mentioned earlier (Table 4). Protease inhibitory activity was checked for five different (Molar concentration) of HCl (0.02M, 0.04M, 0.06M, 0.08M & 0.1M). Bacterial strain M15 showed highest (14.2U/ml) inhibitory activity (U/ml) at 0.02M HCl concentration against trypsin and least (0.2U/ml) value at 0.1M concentration. Bacterial strain M15 showed highest (24.3) Protease inhibitory activity (U/ml) at 0.02M HCl concentration against pepsin and least (0.7U/ml) value at 0.1M concentration.

Table 4
Protease Inhibitory activity of `*Bacillus subtilis*-M15 against Pepsin and Trypsin with Acid Treatment (HCl)

HCl Concentration Molar Solution (M)	Protease Inhibitory activity with Trypsin (U/ml)	Protease Inhibitory activity with Pepsin (U/ml)
0.02	14.2	24.3
0.04	12	19.5
0.06	7.5	10.1
0.08	3.1	5.7
0.1	0.2	0.7

Effects of protease treatment on protease inhibitory activity were calculated against trypsin (Alkaline Protease) and pepsin (Acidic Protease) through a standard assay. Protease inhibitory activity was checked for five different protease concentrations (0.2%, 0.4%, 0.6%, 0.8%, 1%). Bacterial strain M15 showed highest (14.5U/ml) inhibitory activity (U/ml) at 0.2% protease concentration against trypsin and least (8.3U/ml) value at 1% protease concentration (Table 5). Bacterial strain M15 showed highest (23U/ml) inhibitory activity (U/ml) at 0.2% protease concentration against pepsin and least (9.7U/ml) value at 1% protease concentration.

Table 5
Protease inhibitory activity of `*Bacillus subtilis*-M15 against Pepsin and Trypsin with Protease Treatment

Protease Concentration (%)	Protease Inhibitory activity with Trypsin (U/ml)	Protease Inhibitory activity with Pepsin (U/ml)
0.2	14.5	23
0.4	12	20.1
0.6	11.3	18.3
0.8	10.8	12.1
1	8.3	9.7

Protease inhibitory activity of Lopinavir/Ritonavir tablets USP/ compremies USP 200mg/50mg Mylan (Synthetic Protease Inhibitor of HIV-AIDS, Cancer and HCV) was calculated from Koiwa[8] standard assay against trypsin (Alkaline protease present in the cancerous cell) and pepsin (Acidic protease present in HIV-AIDS & HCV). The value of protease pnhibitory activity of Lopinavir/Ritonavir (synthetic PI) was compared with protease inhibitory (PI) activity of novel bacterial Strains of *Bacillus subtilis*-M15

(Table 6). The protease inhibitory activity of the crude extract was 14.2U/ml, the PI activity of the purified sample was 89U/ml, and the PI activity of Lopinavir/Ritonavir was 2446U/ml. In contrast, the PI activity of the immobilized enzyme was 2581U/ml against trypsin. The protease inhibitory activity of the crude extract was 22.3U/ml, PI activity of the purified sample was 120U/ml, and protease inhibitory activity of Lopinavir/Ritonavir was 3360U/ml. In contrast, the PI activity of the immobilized enzyme was 2411U/ml against pepsin.

Table 6
Protease Inhibitory activity of `*Bacillus subtilis*-M15 against Pepsin and Trypsin with Ritonavir (Synthetic Protease Inhibitor)

Bacterial Strain (M15)	**Protease Inhibitory Activity of Crude Extract (U/ml)**	**Protease Inhibitory Activity of the Purified Sample (U/ml)**	**Protease Inhibitory Activity of Immobilized Sample (U/ml)**	**Protease Inhibitory Activity of Ritonavir (Synthetic PI) (U/ml)**
Bacillus subtilis-**M15 (Trypsin)**	14.2	89	2581	2446
Bacillus subtilis-**M15 (Pepsin)**	22.3	120	3360	2411

Inhibitory activity of *Bacillus subtilis*-M15 was calculated against pepsin. Seven different Ion-exchange resins were used to immobilize partially purified protease inhibitors. The figure clearly described the efficacy of ion exchange resin through their enhanced values. The first column represented the crude sample (PI), the second column represented the partially purified sample (PI), and the third column represented the increased values of a sample (PI). Seven ion exchange resins, Lewatite, Dowex 80, Dowex66, Duolite, Amberlite, IR100 and IR401, were represented by the following symbols S1, S2, S3, S4, S5, S6 and S7, respectively. Duolite had shown the highest value (3360U/ml), whereas Lewatite had shown the minimum value (2024U/ml) in a bar chart (Fig. 2).

Discussion

Many researchers, scientists, and authors have practiced the different immobilization methods for different enzymes[21–23]. Different techniques of immobilization were adopted by Beshay and Abd-El-Haleem[24,25]. Effects of hydrogen peroxide were checked on different bacterial strains, and their activity was compared on different concentrations of hydrogen peroxide from 1% v/v to 5%v/v. Inhibitory activity of strain M15, *Bacillus subtilis* M15, was highest against trypsin at 4% v/v. Inhibitory activity of strain *Bacillus subtilis* M15 was highest against pepsin at 3% v/v. Effects of hydrogen peroxide were checked on different bacterial strains, and their activity was compared on different concentrations of hydrogen peroxide from 1% v/v to 5%v/v. It was observed through studies conducted by Bijina *et al.*[26] the effect of

hydrogen peroxide and dimethyl sulfoxide on inhibitory protease activity was negative. On increased concentration of oxidizing agents (Hydrogen peroxide and Dimethyl sulfoxide), there was a decreasing trend of inhibitory activity. Inhibitory activity did not show any significant change by lowering the concentration of oxidizing agent (Hydrogen peroxide and Dimethyl sulfoxide). Effects of dimethyl sulfoxide was checked on different bacterial strains and their activity was compare on different concentration of Dimethyl Sulfoxide from 1% v/v to 5%v/v. Inhibitory activity of *Bacillus subtilis* M15 was highest against trypsin at 2% v/v. Inhibitory activity of strain M15 was highest against Pepsin at 3% v/v. Effects of Dimethyl sulfoxide was checked on different bacterial strains and their activity was compare on different concentration of Dimethyl sulfoxide from 1% v/v to 5%v/v. It was reported by Bijina *et al.*,[26] that, in case of Hydrogen peroxide, there was abrupt loss of inhibitory activity at a concentration above 1%. In case of DMSO, there was gradual decrease in inhibitory activity on increase concentration of DMSO from 1–5%. At 1.4% of Hydrogen peroxide, the complete inactivation of inhibitory activity was observed. At 6% of DMSO, the complete inactivation of inhibitory activity was mentioned.

Results obtained from Yang and Rhee[27] deducted that the ionic binding was a better source of immobilization with higher inhibitory activity and increased mechanical stability; for Amberlite IR-4B, activity was 402U per gram of support, while for Amberlite IRC-50, activity was 600U per gram of support. Inhibitory activity of bacterial strain M2 was calculated. Seven different ion exchange resins were used to immobilize partially purified protease inhibitors. The figure clearly described the efficacy of ion exchange resin through their enhanced values. The first column represented the crude sample (PI), the second column represented the partially purified sample (PI), and the third column represented the increased values of the sample (PI). Seven ion exchange resins, Lewatite, Dowex 80, Dowex66, Duolite, Amberlite, IR100 and IR401, were represented by the following symbols S1, S2, S3, S4, S5, S6 and S7, respectively. Amberlite had shown the highest value (41.3U/ml), whereas Lewatite had shown the minimum value (21.2U/ml) in the bar chart. It was reported by Minovska *et al.*[20] that the activity of the enzyme was increased up to 3.3 folds for Amberlite IRC-50.

Ritonavir is a potential PIs already available in the market, and its inhibitory activity was compared with the inhibitory activity of bacterial strain M15 *Bacillus subtilis*. Ritonavir is a commercially used anti-cancerous, anti-HCV & anti-AIDS drug and a synthetic protease inhibitor. The growth of human myeloid leukemia cells showed degenerated trend while using HIV-1 protease inhibitors. His research concluded that PIs like Ritonavir, Indinavir and Saquinavir was used as inhibitory agents against DU145 and PC-3 androgen-independent prostate cancer growth. Research conducted by Beshay *et al.*,[24], described that Amberlite IRC 50 had an inhibitory activity of 42.3U/ml. Amberlite CG-120 (NA) type III had an inhibitory activity of 31.2 U/ml, and Amberlite CG-4B (OH) type I had 28.4 U/ml. Studies conducted by Minovska *et al.*[20] described that Lipase enzyme was immobilized on Amberlite IRC-50 (H) with the activity of 40.3U/ml, Amberlite IR-4B (OH) with the activity of 75.9U/ml.

Conclusion

Among twenty-five bacterial strains, protease inhibitory activity (U/ml) of *Bacillus subtilis*-M15 tends to degrade acidic and alkaline protease. The immobilized form of the strain has a high potential to break down the pepsin and trypsin. Then protease inhibitory activity of Lopinavir/Ritonavir Tablets USP/ Compremies USP 200mg/50mg (Mylan) was compared with Protease inhibitory activity of identified bacterial Strains *Bacillus subtilis*-M15. Then it is concluded based on results that immobilized bacterial strains have very much higher protease inhibitory values (U/ml) than commercially available medicine on the market. Finally, a novel biological anti-viral drug, which is an immobilized protease inhibitor of *Bacillus subtilis*-M15, having strong acidic protease Inhibitory activity, has proved to lower the concentration of pepsin and pepsin resemble HIV-1 protease based on its 3D structure. Due to the acidic nature of pepsin, the same strains may be used against HCV. Hence, due to the biological nature of finally identified bacterial strains, these may have few side effects compared to already available chemical formula-based medicine like Lopinavir/Ritonavir Tablets.

Declarations

Author contribution statement: MI conducted research and wrote up the initial draft of the manuscript. BN, MZ, and MZA provided sources for research work. FH, TM, AIK, and ZI helped in the statistical analysis of data. QA, MAJ, MSH, and MAM helped edit the manuscript. LS, TS, and IA assisted in the final proofreading of the manuscript. All authors reviewed the manuscript.

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Data Availability Statement: All of the data generated from research has been given in manuscript.

References

1. Bode, W. & Huber, R. Natural protein proteinase inhibitors and their interaction with proteinases. EJB Reviews, 43–61 (1993).
2. Imada, C. Enzyme inhibitors and other bioactive compounds from marine actinomycetes. Antonie Van Leeuwenhoek **87**, 59–63 (2005).
3. Rich, D. H. Evaluation of Enzyme Inhibitors in Drug Discovery: A Guide for Medicinal Chemists and Pharmacologists. Robert A. Copeland. Hoboken, NJ: John Wiley & Sons, Inc., 2005, 271 pp., $84.95, hardcover. ISBN 0-471-68696-4.(Book web site http://www. chipsbooks. com/evalenz. htm). *Clinical Chemistry* **51**, 2219–2220 (2005).
4. Hamilton, S. C., Farchaus, J. W. & Davis, M. C. DNA polymerases as engines for biotechnology. Biotechniques **31**, 370–383 (2001).
5. Rawlings, N. D. Peptidase inhibitors in the MEROPS database. Biochimie **92**, 1463–1483 (2010).
6. Sabotič, J. & Kos, J. Microbial and fungal protease inhibitors—current and potential applications. Applied microbiology and biotechnology **93**, 1351–1375 (2012).

7. Ussuf, K., Laxmi, N. & Mitra, R. Proteinase inhibitors: plant-derived genes of insecticidal protein for developing insect-resistant transgenic plants. Current Science, 847–853 (2001).
8. Koiwa, H., Bressan, R. A. & Hasegawa, P. M. Regulation of protease inhibitors and plant defense. Trends in plant science **2**, 379–384 (1997).
9. Imada, C. Enzyme inhibitors of marine microbial origin with pharmaceutical importance. Marine Biotechnology **6**, 193–198 (2004).
10. Dash, C., Kulkarni, A., Dunn, B. & Rao, M. Aspartic peptidase inhibitors: implications in drug development. Critical reviews in biochemistry and molecular biology **38**, 89–119 (2003).
11. De Clercq, E. Anti-HIV drugs: 25 compounds approved within 25 years after the discovery of HIV. International journal of antimicrobial agents **33**, 307–320 (2009).
12. Newman, D. J., Cragg, G. M. & Snader, K. M. Natural products as sources of new drugs over the period 1981 – 2002. Journal of natural products **66**, 1022–1037 (2003).
13. Koehn, F. E. & Carter, G. T. The evolving role of natural products in drug discovery. Nature reviews Drug discovery **4**, 206–220 (2005).
14. Borowitzka, M. A. Microalgae as sources of pharmaceuticals and other biologically active compounds. Journal of applied phycology **7**, 3–15 (1995).
15. Witvrouw, M. & De Clercq, E. Sulfated polysaccharides extracted from sea algae as potential antiviral drugs. General Pharmacology: The Vascular System **29**, 497–511 (1997).
16. Schaeffer, D. J. & Krylov, V. S. Anti-HIV activity of extracts and compounds from algae and cyanobacteria. Ecotoxicology and environmental safety **45**, 208–227 (2000).
17. Goenaga, D. *et al.* Molecular determinants of Grb14-mediated inhibition of insulin signaling. Molecular endocrinology **23**, 1043–1051 (2009).
18. Kurokawa, K. *et al.* Comparative metagenomics revealed commonly enriched gene sets in human gut microbiomes. Dna Research **14**, 169–181 (2007).
19. Classics Lowry, O., Rosebrough, N., Farr, A. & Randall, R. Protein measurement with the Folin phenol reagent. J biol Chem **193**, 265–275 (1951).
20. Minovska, V., Winkelhausen, E. & Kuzmanova, S. Lipase immobilized by different techniques on various support materials applied in oil hydrolysis. Journal of the Serbian Chemical Society **70**, 609–624 (2005).
21. Subba Rao, C., Madhavendra, S., Sreenivas Rao, R., Hobbs, P. J. & Prakasham, R. Studies on improving the immobilized bead reusability and alkaline protease production by isolated immobilized Bacillus circulans (MTCC 6811) using overall evaluation criteria. Applied Biochemistry and Biotechnology **150**, 65–83 (2008).
22. Anwar, A., Qader, S. A. U., Raiz, A., Iqbal, S. & Azhar, A. Calcium alginate: a support material for immobilization of proteases from newly isolated strain of Bacillus subtilis KIBGE-HAS. World Applied Sciences Journal **7**, 1281–1286 (2009).

23. Kumari, D., Sharm, N., Pandove, G. & Achal, V. Alkaline protease production by immobilized cells of Bacillus pumilis MTCC 2296 in various matrices. Life Sci J **6**, 8–10 (2009).
24. Beshay, U., Abd-El-Haleem, D., Moawad, H. & Zaki, S. Phenol biodegradation by free and immobilized Acinetobacter. Biotechnology letters **24**, 1295–1297 (2002).
25. Abd-El-Haleem, D., Beshay, U., Abdelhamid, A. O., Moawad, H. & Zaki, S. Effects of mixed nitrogen sources on biodegradation of phenol by immobilized Acinetobacter sp. strain W-17. African Journal of Biotechnology **2**, 8–12 (2003).
26. Bijina, B. *et al.* Protease inhibitor from Moringa oleifera leaves: Isolation, purification, and characterization. Process Biochemistry **46**, 2291–2300 (2011).
27. Yang, D. & Rhee, J. S. Continuous hydrolysis of olive oil by immobilized lipase in organic solvent. Biotechnology and bioengineering **40**, 748–752 (1992).

Figures

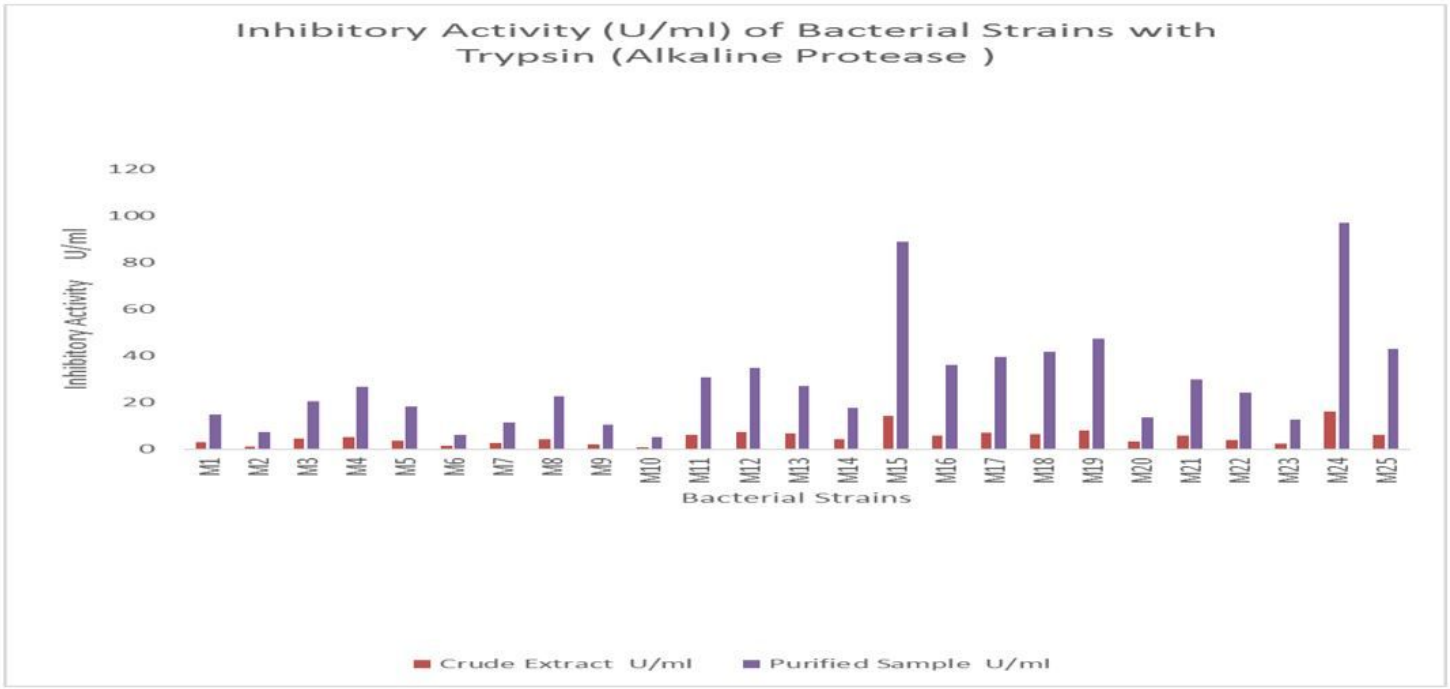

Figure 1

Protease Inhibitory activities (U/ml) of 25 bacterial strain

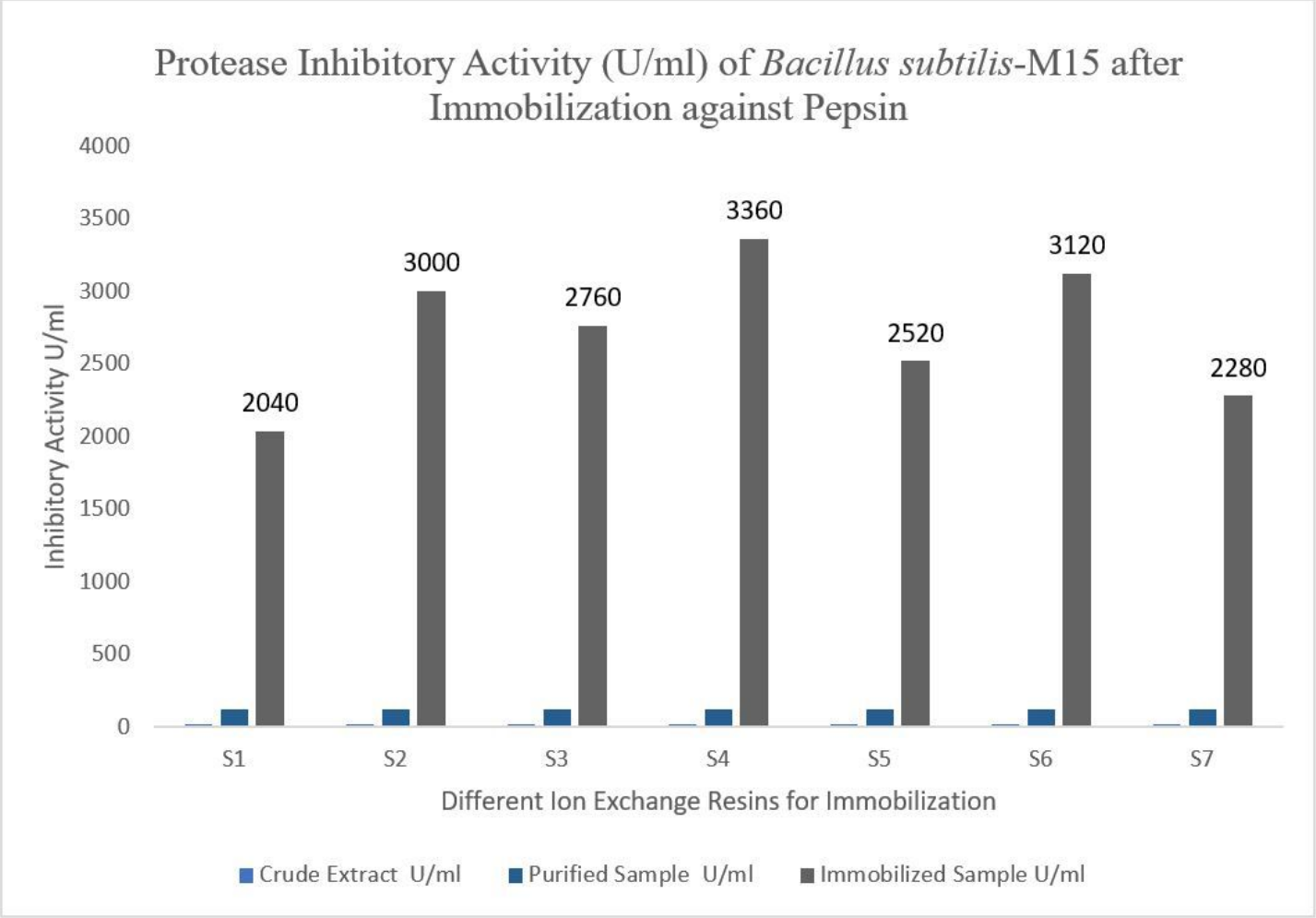

Figure 2

Protease Inhibitory activity (U/ml) of *Bacillus subtilis* M15 for crude, purified and immobilized sample