

A Medium for Facilitating Hepatitis B Virus Detection and Replication of the Virus

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ABSTRACT

In this study, we tested a chemical mixture causing hepatitis virus to grow in excessive amounts. We obtained this chemical mixture by disintegrating albumin and invertase enzymes separately in supercritical environments, using acidic methanol as solvent. Then we used this mixture as a medium for hepatitis B virus. The chemical mixture caused the hepatitis B virus to multiply almost 80-100 times after 120 minutes. The real surprising result of the study occurred after carrying out bacterial tests with the same chemical mixture, its effect on cancer cells were also investigated. But no effect was observed neither on cancer cells, nor on bacteria. In other word, we developed a specific medium for the hepatitis virus. In this way, we decided that a method that can reduce the detection limit of PCR device to 1 IU/mL could be developed. This study will be an important milestone in developing vaccines against viruses as well.

INTRODUCTION

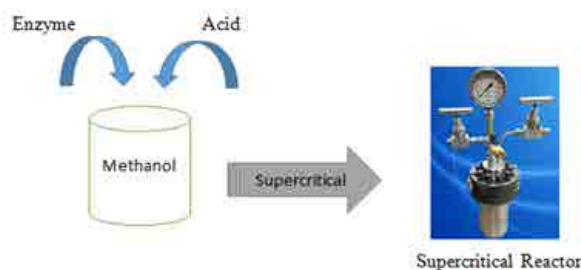
Hepatitis B is a virus that causes liver inflammation. The virus is transmitted through blood and body fluids and sexual contact. The disease caused by the virus and called with its name is divided into two categories, namely acute and chronic. Acute Hepatitis B virus infection is a short-term disease that occurs within the first 6 months after exposure to the virus. Symptoms of the disease are briefly: (a) fever, fatigue, anorexia, nausea and/or vomiting, (b) jaundice (yellow skin or eye color, dark urine, clay-colored stool), (c) muscle, joint, and stomach pain. Chronic Hepatitis B virus infection, on the other hand, may result in bad consequences such as liver damage (cirrhosis), liver cancer and death if the virus cannot be destroyed for more than 6 months from the moment it enters the body. As can be seen, hepatitis virus is one of the most resistant and dangerous viruses [1,2]. Early detection and diagnosis of this virus is also of special importance. Of course, the most widely used method is PCR method, which stands for Real-Time Polymerase Chain Reaction. It is used very often in the determination of nucleic acid. PCR is a method that can produce quantitative results in a short time by measuring the fluorescent signal that increases simultaneously with the proliferation of nucleic acid. Thanks to the fact that temperature cycles and fluorescence can be read in the same device, it is a method that provides much more practical and faster results than conventional electrophoresis. It is possible to perform the reproduction of species and quantitative and qualitative determinations of reproduced species in the device. However, both the device and the materials used in the application of the method are very expensive. A protocol should be created for each analysis [3]. The exposure of appropriate receptors for a virus species on the cell surface and viral entry are parameters that define, among other things, the host and tissue specificity of a virus and often limit the susceptibility of a cell line to a virus species. This problem is exemplified by HBV. Overcoming these obstacles and facilitating efficient transfer and replication of hepatitis B viruses into non susceptible cells would allow to including those concerning early replication events, such as the generation of intranuclear hepadnaviral DNA [4]. The dream of every analyst is to detect even a single virus in the human body. Currently, many research laboratories have PCR and LOD is 30-10⁵ IU/mL for the hepatitis B virus ("LoD=the lowest amount of analyte (measured) in a sample that can be detected with (stated) probability, although perhaps not quantified as an exact value"). According to WHO, hepatitis B DNA load is given as "IU/mL", which is known as the conversion coefficient

to the Copies/ml. According to NIH, 100,000 Copies /ml is the “clinically significant” level. Linear analysis between 25-10⁸ IU/mL is rational. However, the results below 100 IU/mL are still not very healthy. At the same time, vaccine development studies are carried out with weakened viruses. This study will be an important milestone in developing vaccines against viruses. At the moment, Hepatitis B vaccine is mandatory in many countries in the world. Hepatitis B virus vaccine is obtained using recombinant DNA technology, by purifying the surface antigen of the virus after yeast expression and abundant production. These vaccines should contain 10-40 µg/mL surface antigen. This vaccine is also produced with double antigens including inactivated hepatitis A virus. If the vaccine is in prophylaxis after hepatitis contact, immunization with active hepatitis vaccine and passive hepatitis immunoglobulin has been found to be high [5]. Therefore, in order to produce the vaccine, it must be produced in high amounts at the yeasting stage, which is a time-consuming process. For this reason, the significance of the chemical mixture we produce is increasing very seriously. Therefore, in this study, we tried to develop a medium to facilitate the detection of a resistant virus such as hepatitis B. The details of the study are as follows.

MATERIALS AND METHODS

Albumin and invertase enzymes were purchased from Sigma Aldrich. 0.4 g of albumin was dissolved in 30 mL of methanol, about 400 µL of concentrated HI acid was added thereto and mixed. Then the solution was transferred to the reactor. In the reactor, albumin was disintegrated at supercritical point (at 224°C and 65 atm). After the reaction, the obtained product was filtered. The filtered liquid was dried in a vacuum oven. About 0.04 g of the resulting solid was taken and dissolved in 50 mL of pure water in an ultrasonic bath. The insoluble part was filtered again, and the filtrate was used. The same procedures were repeated for the invertase enzyme as well (Figure 1).

Figure 1. Preparation of samples.



Mueller Hinton Broth (MHB) were purchased from Merck (Merck KGaA, Germany). The microorganisms of *Escherichia coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 29213), *Aspergillus flavus* (ATCC 9170) and *Aspergillus niger* (ATCC 6275) were obtained from the cultural collection of the Microbiology Laboratory of Cumhuriyet University Research Hospital and the ampicillin disk was from Oxoid™ (Thermo Fisher Scientific, USA)^[6].

Hepatitis test

Twenty microliters of HBV DNA positive sera (10⁷ IU/ml) (Sigma Aldrich) was added to 980 µl of each sample. The samples were prepared in triplicate and kept for 90, 120, 180 minutes at room temperature. Twenty microliters of HBV DNA positive sera (10⁷ IU/ml) was inoculated to 980 µL of PBS as control, which were also prepared in triplicate and kept for 90, 120, 180 minutes at room temperature. HBV DNA levels of the samples and the controls were measured by real time PCR method (Qiasymphony Qiagen, Germany). Analytical sensitivity and linear range of HBV DNA test was 10.2 IU/ml, and 31.6-2.1 × 10⁷ IU/ml respectively. Each experiment was performed three times and in triplicate.

Determination of Minimum Inhibition Concentration (MIC)

To find the lowest concentration of the DRUG that killed at least 99.99% of the initial number of microorganisms (MIC), the macro broth method was used with slight modification. 25 µL (1 × 10⁸ cfu/mL) of each microorganism cultures, prepared as described above, was mixed with 3 mL of MHB and diluted with different amount of extracts (5 mL; 2,5 mL; 1,25 mL); the mixtures were incubated for 30, 60 and 90 minutes at 37°C and 30°C for the bacteria and molds, respectively. After the incubation, the lowest concentration of the extracts with no microbial growth was set as MIC. Ampicillin was used as the positive control. To determine fluconazole MICs using spectrophotometric kinetic assay, the growth percentage was calculated for each drug concentration using the following equation: growth % = ((OD₄₀₅ of wells containing the drug / OD₄₀₅ of the drug-free well) × 100) after removing background ODs (ODs of microorganism-free wells). The highest OD (OD_{max}), time at mean maximum OD, changes in ODs (delta OD = OD_{final} - OD_{initial}) for each well, and inhibition percentage values were calculated Using Microplate Data Collection and Analysis Software (Bio-Tek Instruments). MIC curves and MIC curve interpolations for each organism were obtained using these values^[7].

Reproduction of cells

In cell culture studies, suitable conditions and necessary medium must be provided for the cells to live and reproduce *in vitro*

environment. MDA-MB-231 (breast adenocarcinoma) cells were reproduced in DMEM medium containing L-Glutamine, 10% FBS, 1% penicillin+streptomycin, 5% CO₂, at 37°C, in T-25 and T-75 flasks, in the incubator. Cells were planted in 24-well plates, and after 24 hours 70% occupancy was observed. The prepared drug concentrations were applied to the cells. After 72 hours, the medium was removed from the wells. Cell lysis solution was added. Then the cells in the wells were disintegrated by ultrasonication. They were put in 96-well plates and Hoechts 33258 solution was added. Measurements were made with multiplate reader. Analyzes were conducted according to DNA standard measurements and DNA quantity-graphs were plotted for each.

The HBV DNA in the supernatant was treated with proteinase K digest (2.5 µg/ml in 100 mM Tris HCl, pH 7.5, 12.5 mM EDTA, pH 8.0, 150 mM NaCl, and 1% SDS) for 4 h at 65°C.

GC/MS analysis

The component analysis of the cleavage products was performed by GC/MS QP2010 Gas Chromatograph Mass Spectrometer (Shimadzu). The analysis was based on the methods set in the previous literature. In the GC part; Column oven temperature was set as 100°C, injection temperature as 275°C, and sampling time as 0.5 minute. In the MS part; Ion source temperature was set as 280°C, interface temperature as 280°C, and solvent cut time as 2.55 minutes. These parameters were measured as in the literature [8,9].

RESULTS

Unlike other tests, 0.04 g of both invertase and albumin cleavage products were taken and dissolved in 50 methanol in an ultrasonic bath. The solution was filtered on filter paper. The filtrate was used for GC/MS analysis. Figures 2a and 2b and Tables 1a and 1b show GC/MS Chromatogram of Albumin and Invertase.

Figure 2a. GC/MS Chromatogram of Albumin.

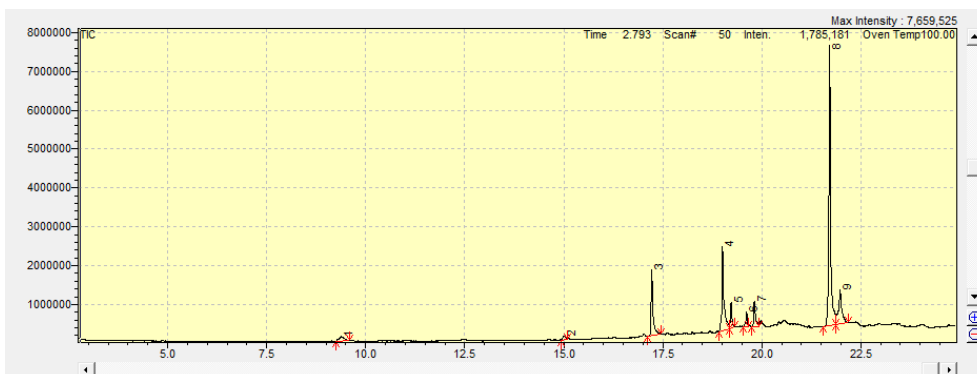


Figure 2b. GC/MS Chromatogram of Invertase.

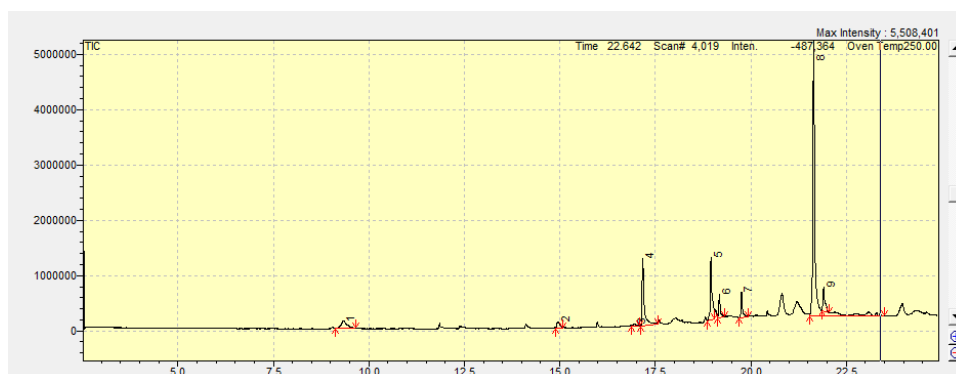


Table 1a. GC/MS results of Albumin.

Peak #	Ret. Time	Start Tm	End Tm	m/z	Area	Area%	Height	Height%	A/H	Mark	Name
1	9.382	9.25	9.59	TIC	950981	1.98	110604	0.8	8.6	-	Decanoic acid, methyl ester
2	15.001	14.93	15.1	TIC	428911	0.89	81370	0.59	5.27	-	Methyl tetradecanoate
3	17.222	17.105	17.45	TIC	5690081	11.82	1686300	12.19	3.37	-	Hexadecanoic acid, methyl ester

4	19.008	18.905	19.19	TIC	7845654	16.3	2156156	15.58	3.64	V	9-Octadecenoic acid(Z)-methyl ester
5	19.225	19.185	19.32	TIC	1591260	3.31	657116	4.75	2.42	V	Methyl stearate
6	19.618	19.53	19.74	TIC	1022830	2.13	375508	2.71	2.72	V	(E)-9-Octadecenoic acid ethyl ester
7	19.812	19.735	19.92	TIC	2158752	4.49	662857	4.79	3.26	V	Octadecanamide
8	21.714	21.555	21.87	TIC	23199211	48.19	7211381	52.12	3.22	-	9-Octadecanamide,(Z)-
9	21.977	21.87	22.2	TIC	5243585	10.89	894918	6.47	5.86	V	Octadecanamide

Table 1b. GC/MS results of invertase.

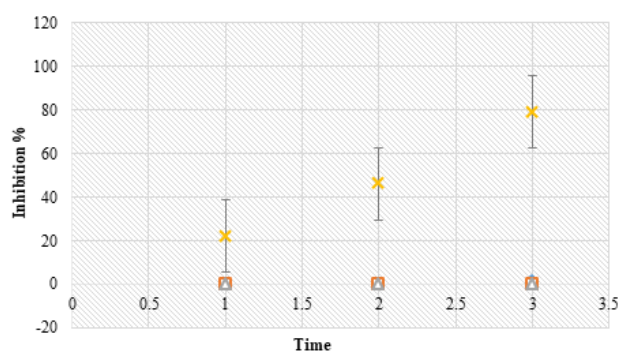
Peak #	Ret. Time	Start Tm	End Tm	m/z	Area	Area%	Height	Height%	A/H	Mark	Name
1	9.331	9.115	9.66	TIC	1542470	4.29	137486	1.51	11.22	SV	Decanoic acid, methyl ester
2	14.964	14.89	15.08	TIC	623126	1.73	104781	1.15	5.95	-	Tetradecanoic acid, methyl ester (CAS)
3	16.966	16.885	17.095	TIC	208761	0.58	37649	0.41	5.54	V	9-Hexadecanoic acid, methyl ester, (z)-
4	17.172	17.11	17.57	TIC	4637109	12.89	1207644	13.25	3.82	-	Hexadecanoic acid, methyl ester
5	18.957	18.86	19.06	TIC	4011680	11.16	1142972	12.54	3.51	V	9-Octadecenoic acid, methyl ester, (E)-
6	19.177	19.13	19.31	TIC	1143497	3.18	424609	4.66	2.69	V	Methyl stearate
7	19.758	19.7	19.935	TIC	1243637	3.46	448789	4.92	2.77	-	Octadecanamide
8	21.65	21.55	23.49	TIC	20987181	58.36	5187696	56.9	3.74	SV	9-Octadecanamide,(Z)-
9	21.911	21.86	22.06	TIC	1565388	4.35	424474	4.66	3.69	T	Octadecanamide

As can be seen in Figures 2a and 2b and Tables 1a and 1b the list of chemicals with 95% and more matching were given from the GC/MS library of cleavage products. Both substances were found to break down into carboxylic acid esters and straight chain amides. Considering the structure of invertase and albumin, they were expected to give these products after a series of cycle mechanisms at high temperature in the acidic environment in which they are put into reaction.

Bacterial test

All dilutions were performed by comparing the optical density of the tubes where the microorganism load was not reduced. The microorganism introduced into the medium was expected to inhibit the chemicals (ampicillin), such as the main antibiotic agent in the control group (It cannot be called as antimicrobial/antifungal unless it shows an inhibition over 10%) [10]. An antimicrobial agent is expected to reduce the burden of microorganisms in the environment after a certain period of time, which can be measured in many different ways and the accuracy of the result can be checked. As a result, the used sample did not act as an antimicrobial and antifungal agent, in Figure 3.

Figure 3. Representation of inhibition test. Note. ● 5 ml; ■ 2.5 ml; ▲ 1.25 ml; ✖ control.

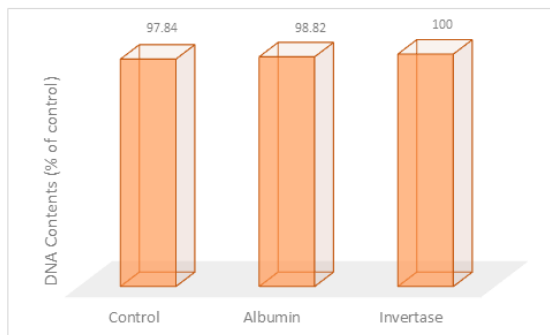


Viruses are very small and don't metabolize by themselves, therefore, they don't have the ability to reproduce on their own. They enter into the cells by force and use them to produce new viruses. They then keep proliferating rapidly in the patient's body by destroying the host cell. Antibiotics have no effect on them. On the other hand, bacteria are a living small organism that consists of a single cell. They can survive in any environment such as air, water, soil, etc. and reproduce very quickly. Antibiotics prevent the growth of bacteria [11]. Unlike the treatment of "pneumonia", which is a bacterial disease that can be treated using antibiotics, an antiviral agent should be preferred in the treatment of "Viral Hepatitis", which is a disease caused by viruses [12].

Cancer test

In the study, living cells were accepted as 100% control. No change was observed in the DNA contents of the cells compared to the control [13]. The fluid obtained from its albumin killed more cells than any other substance, whereas all substances kill the cancerous cell around 100%, in Figure 4. The chemical mixture obtained did not show much effect on cancer cells. Because this mixture does neither have a toxic effect on cancer cells, nor supports the proliferation of cancer cells. It is understood that it does not have an effect on the cell death mechanism in a molecular sense and does not cause a change in the mitochondrial pathway.

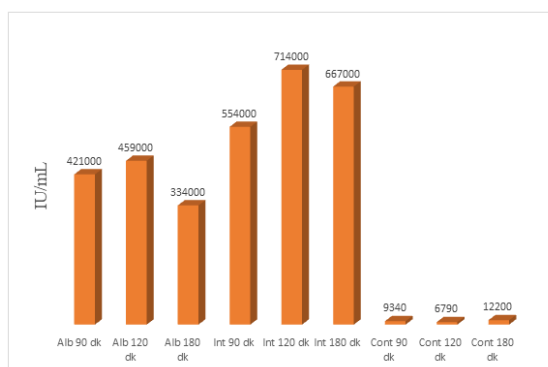
Figure 4. Representation of Cancer Test.



Hepatitis test

The obtained cleavage products were dissolved in pure water in an ultrasonic bath and again filtered on filter paper. The filtrate was used for hepatitis test. As seen in the Figure 5, in the control group 9,340-6,790-12,200 IU/mL hepatitis B virus were detected for 90-120-180 minute-cultivations, respectively, while 554,000-714,000-667,000 IU/mL hepatitis B virus were detected in cleavage products prepared with Invertase enzyme; in other words, the medium leded 100 times more virus growth, especially in 120 minutes. Regarding the cleavage product of Albumin enzyme, 421,000-459,000-334,000 IU/mL hepatitis B virus were detected for 90-120-180 minute-cultivations, respectively, which means that it caused almost 60-70 times more virus growth especially in 120 minutes. Normally, when invertase and albumin enzymes are used as a nutrient medium without putting them in the supercritical environment, they cause 5-10 times more growth than the control group, whereas their products, which were disintegrated in the supercritical environment, caused the growth of almost 100 times more hepatitis B virus. This interesting result is because of the nucleic acid that the virus needs to reproduce. Viruses cannot reproduce on their own, they need other cells. They need nucleotides after being attached to a cell. Glycine/glutamine with a single carbon or amine is needed in the formation of nucleic acids. The concentration (0.003-0.004 g/50 ml) of the amino acids formed by the proteins disintegrated when exposed to high pressure and methanol correspond to an average of 0.5-1 mM. Having 10 times more methanol in a single carbon leads to ionization of the side chain and increases the disintegration of proteins but when faced with high pressure it mostly affects the length. Ultimately, these cleavage products obtained in the supercritical medium, provided the virus a good medium for nucleic acid synthesis (Figure 5).

Figure 5. Representation of Hepatitis Test.



In current study two distinct protein were used: albumin and invertase. Mild acidic conditions in methanol (pH: 5) along with high pressure and temperature breaks the proteins into smaller residues. Cleavage patterns are similar, and this suggest that the disintegration is independent of protein sequence. The process creates key precursors for nucleotide synthesis, protein coat, and single carbon metabolism. As known, in the replication of Hepatitis B virus nucleotides are needed for RNA synthesis. The virus also needs residues for protein coat of the viral genome. The replication is essential in the life cycle of the virus. Glycine, aspartic acid, glutamine, and formate are important for *de novo* pathway. All living organisms seem to have almost identical *de novo* pathways as a result of pyrimidine and purine biosynthesis. The free bases may not be the intermediates of the *de novo* pathways, but they form the intermediates of some salvage pathways. *De novo* pathways share various precursors in the synthesis of purines and pyrimidines. Each pathway type has an amino

acid as an important precursor, namely purines have glycine and pyrimidines have aspartate. The most important source of amino groups is Glutamine, which is used in five different steps of *de novo* pathways. Purine pathways use aspartate as the source of an amino group in two steps. Phosphoribosyl Pyrophosphate (PRPP) is important in both of them, and in these pathways the structure of ribose is retained in the product nucleotide ^[14].

Nucleotide synthesis determine the replication rate and may stop as the synthesis lag behind. Therefore, the cleavage of the proteins supplies the intermediates for salvage pathway and precursor for *de novo* synthesis. Supplying single carbon-formate and protein coat components through the process augments nucleotide content.

This process accelerated the replication effectively because cleavage pattern produced proper precursors and intermediates for Hepatitis B replication. Moreover, the addition of this soup at proper concentration (0.003 g/50 ml) accelerates virus replication cycle as well. Optimization of the process for different viruses may be the subject of clinical studies.

CONCLUSION

In this study, we have disintegrated invertase and albumin enzymes in a supercritical environment and we analyzed the released product by GC/MS. We did not find any serious differences when we performed cancer and bacteria tests of cleavage products. But the results of hepatitis B virus tests were quite surprising. We realized that we produced a medium that caused the virus to reproduce almost 100 times more in 120 minutes. Such a medium can be helpful in the production of algae in bioreactors, in the production of some microbial organisms, in the determination of some disease-causing species and in the rapid generation of their standards. With the product obtained as a result of this study, a medium was developed for Hepatitis B virus. At the same time, it is possible to detect very low amounts of Hepatitis B virus in the human body thanks to a calibration to be created with this medium. At the same time, vaccine development studies are carried out with weakened viruses. This study will be an important milestone in developing vaccines against viruses. Currently, studies have been initiated on the enrichment or increasing the effectiveness of DNA taken from the babies of prospective mothers. In our next study, other characteristics of the product produced by this method will be published.

COMPETING INTEREST

The authors declare that there are no competing interests.

DATA AVAILABILITY

The data of the article is available in public.

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