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Enzymatic Removal of Off-flavors from Apple Juice

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Contaminating microorganisms such as *Actinomycetes*, *Alicyclobacillus*, and *Chlostridium* can generate off-flavors in apple juices. Such bacterial metabolites represent, besides phenol types such as guaiacol and 2,6-dibromophenol, a broad range of other chemicals, for example, geosmin, 2-methylisoborneol, or α-terpineol. A laccase from *Trametes hirsuta* was purified, immobilized, and applied for the selective elimination of off-flavor substances in apple juice caused by microbial contamination. The evaluation using GC-MS showed that enzymatic treatment could reduce the amount of guaiacol and 2,6-dibromophenol in apple juice significantly by 99 and 52%, respectively. Upon addition of mediators, the degradation could be increased and the spectrum of substrates extented. Furthermore, commercial apple juices spiked with off-flavors were treated in a continuous-flow reactor and tested by sensory evaluation.

KEYWORDS: Laccase; off-flavor; apple juice; *Trametes hirsuta*

INTRODUCTION

Apart from the bulk production and processing of food and beverages such as beer, biotechnology widened its application in the food industry by specific processes to increase product quality such as by the generation of flavors. Thereby, enzymes showed their potential in various fields such as modification and bioconversion, biosynthesis, and correction of off-flavors (*1*).

Hydrolysis of macromolecules such as fats, proteins, or carbohydrates increases their impact on flavor. Lipases, proteases, and glutaminases are most commonly used in cheese manufacture and the fermentation of soy, respectively. They are applied successfully to decrease the ripening time of Cheddar cheese (*2*), for initiation of volatile flavors in black tea (*3*), or to improve the quality of soy sauce (*4*).

Biocatalysts exhibit high efficiency in stereo- and regioselectivity compared to classical organic reactions. For example, menthol, an outstanding food additive, is suitable in only one of the possible eight isomeric forms (*5*). Legislation allowing labeling of products as "natural" even when treated enzymatically facilitated the implementation biosynthesis in the food industry due to economical benefits (*6*).

Enzymes have been used for the removal of off-flavors inlcuding the oxidation of limonilin, a bitter compound of grapefruit, with limonoate dehydrogenase, the treatment of ultrahigh-temperature (UHT) milk with sulfhydryl oxidase, or the decomposition of hexanal with cell-free extract of baker's yeast (*1*). Various off-flavors in apple juices can be introduced as metabolites from microorganisms such as *Actinomycetes*, *Alicyclobacillus*, and *Chlostridium*. These bacterial products represent, besides phenol types such as guaiacol and 2,6-dibromophenol, a broad range of other chemicals, for example, geosmin, 2-methylisoborneol, or α-terpineol. Although present only in very small amounts, they are responsible for strong off-flavors described as "musty", "earthy", or "like roots" (*7, 8*). Because most of the microorganisms are spore formers, they may survive even after processing steps such as pasteurization and are regeminate in even in the shelf-stable product (*9*).

Laccases could have a potential to eliminate these off-flavors in apple juice. They are copper-containing polyphenol oxidases that oxidize polyphenols, methoxy-substituted phenols, diamines, and a considerable range of other compounds using molecular oxygen as an electron acceptor (*10*). Laccases show high potential for application in the food (*11*), textile (*12*), and pulp and paper industries (*13*).

Although there is only little information about the treatment of apple juice with laccases, the stabilization of apple juice either by a single enzymatic process (*14*) or in combination with ultrafiltration (*15*) has been investigated. However, biotransformation of the whole spectrum of compounds responsible for off-flavors has not been studied in a systematic approach until now. In this study, a laccase from *Trametes hirsuta* was purified, immobilized, and used for the elimination of off-flavor substances in apple juice, which was evaluated using GC-MS and sensory evaluation.

MATERIALS AND METHODS

Chemicals and Enzyme. 2,3-Dimethylpyrazine (95%+), [(1*S*)-*endo*]-(−)-borneol (99%), *m*-anisaldehyde (97%), *p*-anisaldehyde (>98%),

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Table 1. Purification Steps of the 38 kDa Laccase from *T. hirsuta*

step	recovery		specific activity		
	protein, mg L^{-1}	activity, nkat mL^{-1}	nkat mg^{-1} of protein	purification factor	yield, %
diatom earth	7000	607	86	nd[a]	nd
Q FF	158	41	259	>3.0	7
gel filtration	140	40	285	1.1	98

[a] Not determined.

1-octen-3-ol (98%), 3-octanone (>97%), fenchylalcohol (97%), α-terpineol (96% +), guaiacol (98%), 3-isopropyl-2-methoxypyrazine (97%), 2,6-dibromophenol (99%), 2,2-azinobis(3-etylbenzothiazoline-6-disulfonic acid (ABTS), 1-hydroxybenzotriazol (HOBt), 3-aminopropyltriethoxysilane, and glutaric dialdehyde were from Sigma-Aldrich. Coomassie Brilliant Blue reagent was purchased by Bio-Rad (Hercules, CA). All other chemicals and solvents were of analytical grade.

Enzyme Purification. Cultivation of *T. hirsuta* (IMA2002) is described elsewhere (*16*). Purification of the laccase was done in three steps. Crude material from the cultivation was filtered and stirred with diatom earth. After desalting by ultrafiltration using a vivaflow50 (MW_{cutoff} 10000) (Vivascience, Hanover, Germany), anion exchange chromatography was performed, using a Q FF column (Amersham Pharmacia, Uppsala, Sweden) eluting with a four-step (0, 6, 16, and 100% B) gradient with a linear flow rate of 1 mL min^{-1} for 16 column volumes to elute with the final buffer (50 mM acetate, pH 4.3) containing sodium chloride (1.0 M). Collected fractions were analyzed by measuring laccase activity using ABTS as described below. Finally, a gel filtration was performed using a Superdex 75 HR 10/30 column (Amersham Pharmacia, Uppsala, Sweden) with a 200 μL sample volume and a phosphate buffer (0.1 M, pH 6.0) containing sodium chloride (0.1 M) as eluent at a flow rate of 0.5 mL min^{-1}.

Immobilisation of the Laccase. Alumina oxide beads (Sigma) were silanized twice in a 5% (v/v) solution of 3-aminopropyltriethoxysilane in acetone for 24 h at 50 °C and immersed in a 6% (v/v) aqueous solution of glutaric dialdehyde followed by an incubation in 200 mL of a laccase solution (0.4 nkat mL^{-1}, 16 mg L^{-1} protein) for 4 h at room temperature (*17*). Two different diameters of beads were used, 1 and 3 mm, for preliminary studies and in the continuous-flow reactor, respectively.

Laccase Activity and Protein Assay. Laccase activity was determined using ABTS as substrate. Fifty microliters of the sample was mixed with 700 μL of a 5 mM ABTS solution in succinate buffer (25 mM, pH 4.5). The increase in absorbance at 436 nm due to the formation of a green radical was followed, using a Hitachi U-2001 spectrometer (*18*). Optimal conditions for guaiacol degradation were elaborated for two different factors, temperature and pH, at four different levels (30−60 °C and pH 3−6) according to a full factorial experimental design. Therefore, 700 μL of a 1 mM guaiacol solution was incubated with 300 μL of enzyme (40 nkat mL^{-1}) for 3 min at different pH values and temperature. After the desired incubation time, the reaction was stopped by adding 50 μL of a 1 M NaF solution.

The Bio-Rad dye-binding assay, based on the method of Bradford, was used routinely. Coomassie Brilliant Blue reagent (200 mL) was added to 800 mL of sample and mixed thoroughly. After 5 min of incubation at room temperature, the absorbance was measured at 595 nm using a Hitachi U-2001 spectrometer. Protein determinations were carried out in duplicate using bovine serum albumin as standard.

Degradation of Off-flavors. Preliminary studies were performed in a 1.5 mL vial containing 500 μL of the appropriate standard at 10 mg L^{-1}. Different off-flavors compounds, for example, 2,3-dimethylpyrazine, [(1*S*)-*endo*]-(−)-borneol, *m*-anisaldehyde, *p*-anisaldehyde, 1-octen-3-ol, 3-octanone, fenchylalcohol, α-terpineol, guaiacol, 3-isopropyl-2-methoxypyrazine, and 2,6-dibromophenol, were screened for enzymatic degradation, incubating with one bead carrying immobilized laccase with a total activity of 0.01 nkat for 24 h in both water and apple juice. After the desired incubation time, the reaction was stopped by adding 50 μL of a 1 M NaF solution. As blank, standards were incubated with beads without laccase. Further HOBt or ABTS as mediator was elaborated by performing the enzymatic degradation in presence of 10 μL of a 5 mM solution of mediator.

In a second step, 5 mL of apple juice, spiked with 10 mg L^{-1} of the appropriate standard, was incubated in a plugged 50 mL Erlenmeyer flask for 24 h at 50 °C with 2, 4, 6, 8, and 10 pellets, respectively.

For each experiment the degradation of the related off-flavor was evaluated by GC-MS analysis in duplicate as described below.

GC-MS Analysis. Sample preparation using headspace solid phase microextraction (SPME), calibration, identification, and quantification of the target substances and validation of the method are described in detail elsewhere (*7*). In brief, samples (0.5 mL of apple juice diluted with 4.5 mL of water with 2.5 g of Na_2SO_4) were thermostated at 60 °C for 5 min (guaiacol and 2,6-dibromophenol) or 10 min (other substances) and thoroughly stirred. The SPME fiber [50/30 μm DVB/CARTM/PDMS Stable Flex fiber, 2 cm (Supelco)] was exposed into the sample headspace for 30 min (for guaiacol and 2,6-dibromphenol) or 10 min (other substances) and then transferred directly to the injection port of the GC. For the GC-MS measurements a HP G1800A GCD system (Hewlett-Packard) with a cross-linked 5% phenyl methyl siloxane column (column length = 30 m, inner diameter = 0.25 mm, film thickness = 1 μm) and helium as carrier gas were used. For identification and quantification, MS analysis was performed with electron impact ionization (70 eV) either by scanning a mass range mode or by selecting a specific ion mode.

Bulk Treatment in a Reactor. A jacketed glass column (10.5 cm height and 5.0 cm diameter) packed with immobilized laccase and a P-50 pump (Amersham Pharmacia) were used as a continuous-flow reactor. The column was tempered at 50 °C, and the apple juice was treated with a continuous flow rate at 2 mL min^{-1}.

Sensory Evaluation. Seven members of the sensory test panel, who were specifically trained on the off-flavor problem in apple juice, participated in the sensory evaluation. Details on the training procedure are given in ref *8*. In addition to their general qualification as a member of a sensory test panel, the selection of the panellists was performed according to their ability to detect, recognize, and describe potential off-flavors in apple juice.

The sensory evaluation of the apple juice samples treated with laccase was performed for guaiacol and 2,6-dibromophenol because the analytical results showed the greatest influence of the enzyme treatment on these two substances. The sensory thresholds for the two compounds were determined previously with a highly trained panel. The panelists cooperating in this work were part of this panel. The detection thresholds were 0.57 μg L^{-1} for guaiacol and 0.009 μg L^{-1} for 2,6-dibromophenol. The recognition thresholds were at 2 μg L^{-1} for guaiacol and 0.085 μg L^{-1} for 2,6-dibromophenol (*8*).

For the sensory test commercially available apple juice without any detectable off-flavor was used. An aliquot of the apple juice was spiked with 10 μg L^{-1} of either guaiacol or 2,6-dibromophenol to have enough substrate for the enzyme reaction in the sample. These samples and the rest of the apple juice, the so-called "reference juice", were treated with laccase for 24 h at 50 °C.

The sensory evaluation was carried out using triangle tests (*19*) containing two reference juice samples and one sample spiked with guaiacol or 2,6-dibromophenol after the enzymatic treatment. The panelists were asked to detect the odd sample and to describe the sensory properties and differences in relation to the other two samples.

RESULTS

Purification, Characterization, and Immobilization of the Laccase. A three-step purification procedure was developed consisting of desalting and anionic exchange and size exclusion chromatography (**Table 1**). By application of the anionic

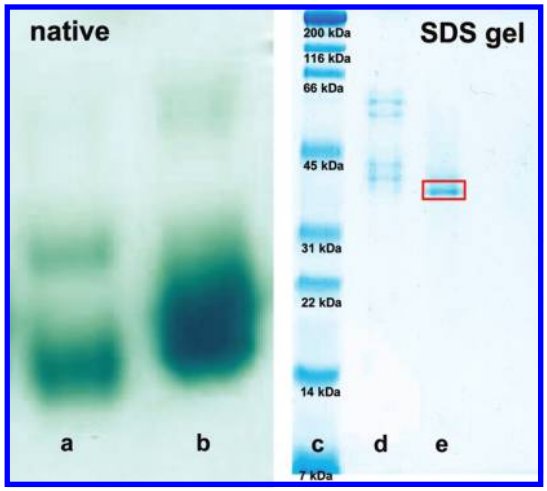

Figure 1. Native gel (left) stained with ABTS and SDS-PAGE (right) of the fractions collected from the anionic exchange chromatography. Lanes: a, fraction with the 61 kDa laccase stained with ABTS; b, fraction with the 38 kDa laccase stained with ABTS; c, molecular weight standards; d, fraction with the 61 kDa laccase stained with Coomassie Brilliant Blue; e, fraction with the 38 kDa laccase from *T. hirsuta* stained with Coomassie Brilliant Blue.

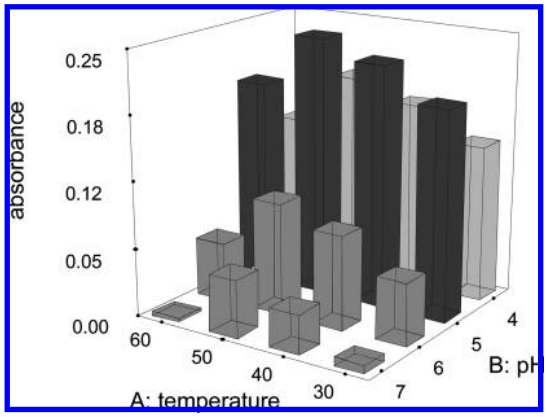

Figure 2. Optimization of the enzymatic degradation of guaiacol by a laccase from *T. hirsuta*.

exchange chromatography, two different fractions exhibiting phenol oxidoreductase activity were found. The resulting enzymes were allocated molecular masses of 38 and 61 kDa via SDS PAGE (**Figure 1**). The latter was of lower amount and showed less activity. After the different purification steps, a recovery of 9% with a 3-fold increase of the specific catalytic activity was obtained for the low molecular mass laccase.

The purified enzyme showed good activity against various phenolic compounds, such as guaiacol and 2,6-dibromophenol, and the optimum conditions were substrate releated. Thereby, the pH optimum varied from 4.0 for DMP to 5.0 for guaiacol and the temperature from 30 °C for DMP to 50 °C for guaiacol (**Figure 2**). A summary of the kinetic data and optima is given in **Table 2**.

An immobilization yield of 48% protein and a retention of 83% of the initial activity resulted in a catalytic capability of 0.04 nkat/mg of dried support. Temperature stability at 70 °C could be increased 5-fold compared to the native enzyme due to immobilization (**Figure 3**). For the reactor, the silica beads loaded with the laccase from *T. hirsuta* could be used for up to nine cycles without significant loss of activity. Immobilization did not show any significant changes in the optima for laccase activity (data not shown).

Table 2. Properties of a 38 kDa Laccase from *T. hirsuta* on Guaiacol and 2,6-Dibromophenol

	optima		kinetic	
substrate	temp, °C	pH	K_M, mM	k_{cat}, s^{-1}
guaiacol	50	5.0	0.21	33.0
2,6-dibromophenol	50	5.0	nd[a]	nd
DMP	30	4.0	0.33	59.0

[a] Not determined.

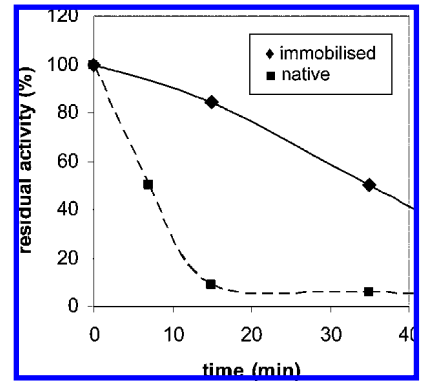

Figure 3. Enzymatic activity of native and immobilized laccase from *T. hirsuta* after incubation at 70 °C at pH 4.0.

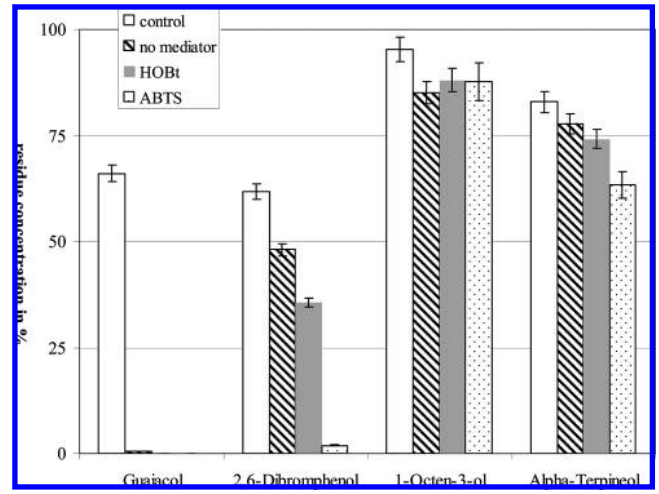

Figure 4. Impact of the mediator on the enzymatic degradation of different off-flavor compounds by a laccase from *T. hirsuta*. The standard solutions were incubated for 24 h at 50 °C in the presence of 0.1 mM mediator. As control, the samples were incubated with beads without enzyme.

Degradation of Off-flavors. From all investigated substances described as off-flavor-causing compounds in apple juice, only the phenolic compounds and α-terpeniol were degraded significantly. In particular, significant reduction of the relative concentrations was measured for guaiacol (99%), 2,6-dibromophenol (52%), and α-terpineol (23%). The enzymatic oxidation was enhanced for all tested off-flavor compounds by the addition of mediator. Thereby, the degradation increased in the order of no mediator, HOBt, and ABTS for all of the substrates (**Figure 4**). Interestingly, the effect (performance) of immobilized laccase on 2,6-dibromophenol in apple juice was increased in the presence of other compounds such as guaiacol (**Figure 5**), treating a sample spiked with a mixture of different off-flavors.

Prior to the enzymatic apple juice treatment in continuous mode, the degradation of off-flavor compounds in apple juice was studied in relation to the amount of beads and time in a batch assay (**Figure 6**).

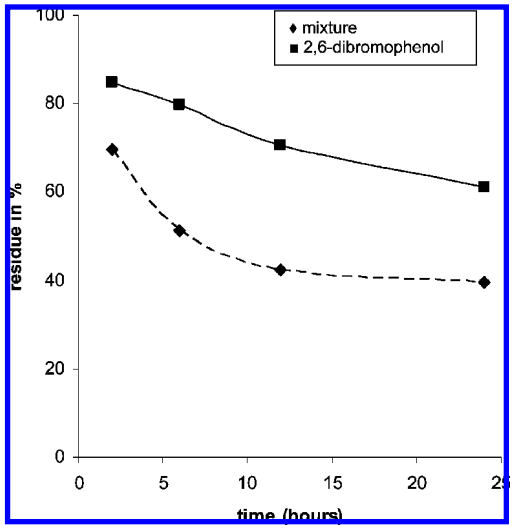

Figure 5. Degradation of 2,6-dibromophenol by a laccase from *T. hirsuta* in apple juice spiked with pure 2,6-dibromophenol and a mixture of different off-flavor compounds, respectively.

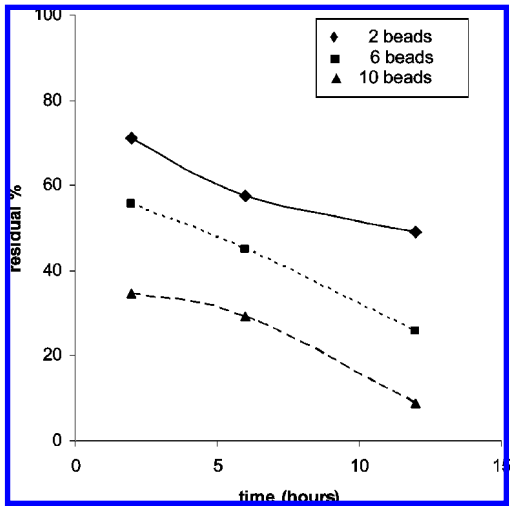

Figure 6. Impact of the number of beads on the enzymatic degradation of guaiacol by a laccase from *T. hirsuta*. The standard solutions were incubated at 50 °C.

Sensory Evaluation. After a continuous enzymatic treatment with an immobilized laccase from *T. hirsuta*, apple juices spiked with 2,6-dibromophenol or guaiacol were evaluated in comparison to nonspiked apple juice as reference, which was also treated with laccase. For the apple juices spiked with 2,6-dibromophenol a statistically significant difference ($\alpha = 0.05$) was observed by the sensory test panel in the triangle test between the spiked and the reference samples. Although the odd sample was detected to be statistically different from the reference samples, the panel was not consistent in the perceived differences between the reference and the treated samples. Two tasters described the odd sample as "medical" or "chemical", the typical characterization of 2,6-dibromophenol. For the other members of the sensory panel no difference between the spiked juice with 2,6-dibromophenol and the nonspiked juice could be detected. These results indicate that the laccase treatment of apple juice containing 2,6-dibromophenol results in a significant reduction of the respective off-flavor.

For the apple juices spiked with guaiacol no significant difference was observed by the panel between spiked and the reference samples, both treated with laccase. Again, the results show that laccase treatment of apple juice tainted with guaiacol may significantly reduce the undesired off-flavor.

DISCUSSION

Purification and Immobilization of the Laccase. Two laccases with different molecular masses, 38 and 61 kDa, respectively, were purified from *T. hirsuta*. The larger enzyme is already described in detail elsewhere (*16*), whereas there is only little information in the literature about laccases with molecular masses below 40 kDa. Although the purified laccase exhibits in general similar properties, it is only 38 kDa large, a rather small enzyme compared to other phenol oxidases described in the literature (60–390 kDa) (*20*). Previously, our immobilization strategy for laccases was successfully applied for dye decolorization (*17*). With 48% yield and 83% retained laccase activity, the immobilization showed a rather good performance compared to other techniques described in the literature (*21, 22*). Besides the improved handling and increased thermostability, a further advantage is the fact that no laccase is added freely to food.

Degradation of Off-flavor Compounds. In the first stage the potential of laccase degradation was studied on different compounds that are described to cause off-flavor in apple juice (*7*). Thereby, only the phenolic compounds, in particular, guaiacol and 2,6-dibromophenol, and α-terpineol were degraded significantly. This was not astonishing, because guaiacol is a well-known laccase substrate and other laccases were also able to oxidize different phenolic compounds in apple juice phenols as well, restricted only by their redox potential (*23*). The degradation of the major compounds as catechin and chlorogenic acid is described in the literature (*24*). However, when used in combination with mediators, the substrate spectrum of laccase can be broadened to include compounds that are not laccase substrates on their own (*10*). Two typical representatives were chosen, ABTS for the electron transmission (ET) mechanism and 1-hydroxybenzotriazole (HOBt) for the hydrogen atom transmission (HAT) mechanism (*25*). In our case best results were obtained with an enzyme mediator system based on electron transmission mechanism using ABTS. Furthermore, the increased degradation of 2,6-dibromophenol in the presence of guaiacol indicates that guaiacol might function as an electron mediator. However, it has to be mentioned that for this particular application a further screening for food-grade mediators would be necessary. Natural compounds such as vanillin or ferulic acid could be nontoxic alternatives with potential for industrial application (*26, 27*).

Apple Juice Treatment. Although the laccase treatment of apple juices was already the subject of previous studies focusing on undesirable color formation and product stabilization, little is found in the literature related to the treatment of off-flavor. Besides the successful removal of off-flavor, the enzymatic treatment resulted also in changing of the main characteristic of apple juice flavor. This may be related to two different reasons. On the one hand, the organoleptic characteristics of the product are affected negatively by simple heating of apple juice up to 50 °C for a longer period (*28, 29*). On the other hand, excessive removal of the phenolic compounds from the apple juice will affect negatively the flavor and taste of the product (*23*).

However, this study clearly demonstrated the potential of an immobilized laccase from *T. hirsuta* to degrade off-flavor compounds with phenolic structure of apple juice in continuous fixed-bed reactors. To increase the selectivity and to reduce

incubation time, the elaboration of optimal treatment conditions might be the subject for further investigation.

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