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# EFFECT OF AMINO ACIDS IN *ALOE* EXTRACT ON PHAGOCYTOSIS BY PERIPHERAL NEUTROPHIL IN ADULT BRONCHIAL ASTHMA

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**The dialyzable material from fresh leaves of *Aloe arborescens* var. *natalensis* was examined in phagocytosis and a phagocytic killing test of *Candida albicans*. Separation of the active component was carried out by chromatography on ion exchange resins, and the activity was detected in the neutral amino acid fraction. The results from the bioassay and comparative study on amino acid compositions in the fractions isolated showed a positive participation by cysteine and proline in phagocytosis. A mixture of cysteine and proline (1:1) significantly enhanced the depressed phagocytosis of neutrophils in adult bronchial asthma.**

**Key words:** *Aloe* — asthma — cysteine — phagocytosis — proline

## INTRODUCTION

Oral administration for six months of an extract of *Aloe arborescens* var. *natalensis* showed efficacy for chronic bronchial asthmatics of various ages as well as intrinsic types. As shown in Table 1 an important finding was that the extract was not efficacious at all for patients who had previously been administered corticosteroid[1]. These findings suggest that the components of the extract may be involved in a restoration of protective mechanisms followed by augmentation of resistance against infection.

In an earlier experiment, screening for phagocytosis and nitroblue tetrazolium chloride (NBT) reduction of neutrophils in adult bronchial asthma were performed. The active components in the nondialysate of *Aloe* extract were isolated and found to be: polysaccharide C (mol. wt., 40,000), a linear polymer of 1–4 O-linked $\beta$-D-mannopyranose and glycoprotein A (mol. wt., 40,000, protein 57%, hexosamine 4%, and carbohydrate 34%) consisting of glucose, mannose, galactose, glucosamine, N-acetylglucosamine and amino acids[2]. During these experiments, phagocytosis-stimulating activity was also found in the dialyzable fraction, suggesting the participation of active component(s) with a small molecular weight. In the earlier study, the NBT reduction test, in which the purgative barbaloin showed a positive response, was used for the bioassay. In the present screening, however, a phagocytic killing test for *Candida albicans* was undertaken instead of the NBT reduction test. This paper deals with the identification of the active component on the basis of this bioassay, and the role of a single amino acid for phagocytosis is discussed.

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**Abbreviations:** NBT nitroblue tetrazolium chloride; PBS phosphate buffered saline

## MATERIAL AND METHODS

*Plant material* The fresh *Aloe* leaves were harvested in June, 1984, at Fukuyama University's herbal garden, green house, where a specimen is available for inspection.

*Methods of Analysis* Thin layer chromatography (TLC) of carbohydrates was done on a cellulose plate (Cellulose $F_{254}$, Merck) with BuOH-pyridin-$H_2O$ (6:4:3) and BuOH-AcOH-$H_2O$ (4:1:5, upper layer) as solvent systems and aniline hydrogenphthalate for detection. TLC of amino acids was performed on a DC Aufolien Kieselgel 60 (Marck) plate and BuOH-EtOH-$CHCl_3$ –28% $NH_4OH$ (4:5:2:8) and EtOAc-BuOH-AcOH-$H_2O$ (6:8:5:8) were used as solvent systems. Gas chromatography (GC) of the methyl ester of acids was performed on a glass column (2 m × 3 mm, I.D.) of chromosorb W (AWDMCS, 60–80 mesh) with 1,4-

Phagocytic index =

$$\frac{\text{number of yeast phagocytized by 100 neutrophils}}{\text{control}}$$

Control: The sample was replaced by 0.05 ml of 0.15M PBS, pH 7.2.

*Phagocytic killing test of C. albicans* This test was performed by a modification of Yamamura's method[5]. Briefly, *C. albicans* was maintained on 1-1.5% agar (Hoechst) slopes containing peptone (Difco) and 2% glucose. Subcultures were made in 1% peptone and 2% glucose broth for 3-4 days at 28°C. The broth cultures gave a homogeneous yeast form of *C. albicans*. Duplicate samples of *C. albicans* ($2.5 \times 10^6$/ml) and human neutrophils were mixed in tubes, each containing 0.2 ml of *C. albicans* ($2.5 \times 10^6$/ml) and 0.1 ml of neutrophils suspended in 0.1 ml of RPMI-1640 solution with 1.25% standard AB serum. To each tube 0.1 ml of the sample dissolved in 0.15 M PBS, pH 7.2, at a concentraiton of 1 to $10^{-5}$ mg/ml was added, and the tubes were incubated under constant rotation for a further 15 min at 37°C. Then, a suspension of 0.1 ml of 1.2% sodium deoxycholic acid and 0.2 ml of deoxyribonuclease (100 μg/ml) was added and incubation under constant rotation continued for a further 15 min at 37°C. Aliquots of 200 μl/well were then transferred to microtiter plate wells to each of which 5 μl of $^3$H-uridine (40 μCi/ml) was added, and the microtiter plates were incubated for a further 60 min at 37°C. Cells associated with radioactivity were then collected onto glass fiber discs with a harvester placed into toluene-2,5-diphenyloxazole scintillation fluid, and counted in an Aloka liquid scintillation counter, Model 653. As the control, RPMI-1640 solution was used instead of the sample. Results were expressed by the index of killed *C. albicans*.

Phagocytic index of killed *C. albicans* =

$$\frac{\text{cpm } (C.\ albicans \text{ and neutrophils})}{\text{cpm (control)}}$$

*Patients* Response to *Aloe* extract by chronic asthmatics was summarized in Table 1. All patients were required to sign a consent form. Assessments were obtained clinically from both patients' impressions and physicians' observations. The clinical trial was done for 24 weeks preceded by a 4 week basal period. Five milliliters of a 20% solution of *Aloe* extract in saline was administered orally twice a day for 24 weeks. Clinical and laboratory assessments were obtained at the beginning and end of the 4-week basal period and at 2-week intervals thereafter. At the conclusion of the 24-week period, the patient was given an option to continue or discontinue treatment by means of the following questionnarie: Would you prefer to: (1) Stop treatment? (2) Continue with the extract from the first 12-week period? (3) Continue with the extract from the second 12-week period? (4) No special preference. Physician evaluation forms were fild at the end of each 2-week period and were completed at the end of the 24-week treatment with reference to the patients' symptom diaries, access to which had been given readily at the beginning of this trial. Physician evaluation was based on the following scheme: (1) No change, (2) Slightly better, (3) Very much better, (4) Slightly worse, (5) Very much worse. In order to fairly evaluate the extract, only response No. (3) was considered positive. There were no adverse effects through the 24-week trial

Table 1 Response to *Aloe* extract by chronic asthmatics

| | | No. of Subjects | No. of Affected cases (%) |
|---|---|---|---|
| Age | 11-30 | 11 | 2(18.2) |
| | 31-50 | 14 | 4(28.6) |
| | 51- | 8 | 5(62.5) |
| Type* | Exogeneous | 12 | 2(16.7) |
| | Endogeneous | 21 | 9(42.9) |
| Corticosteroid** dependence | No | 27 | 11 |
| | Yes | 6 | 0 |
| Total | | 33 | 11(33.3) |

*According to Rackman's classification.

**The patient who does not spend an usual daily life without corticosteroid.

period.

## RESULTS

*Effect of the dialysate on phagocytosis and a phagocytic killing test of C. albicans*

The pale yellow dialysate which was obtained from the supernatant by use of a visking tube, gave a positive response to these phagocytosis assays. Since phenolic substances, such as barbaloin, are contained in this fraction, it was separated into fractions by chromatography on nonionic porous resin, Amberlite XAD-2 (Fig. 1).

*Effect of nonphenolic and phenolic substances on phagocytosis and a phagocytic killing test of C. albicans*

Nonphenolic substances produced a positive response in both phagocytosis assays, but phenolic substances produced a suppression in both assays at the dilution of the sample and 0.15 M PBS (1:1). The amino acid analysis of nonphenolic substance showed the following composition (%): Asp (10.9), Thr (1.9), Ser (2.7), Glu (9.8), Gly (6.9), Ala (7.9), Cys (3.8), Val (15.4), Met (1.9), Ile (2.2), Leu (7.3), Tyr (10.0), Phe (5.0), Orn (4.2), Lys (2.3), His (3.6), Arg (2.8), Pro (1.4). (Table 2)

*Effect of the precipitate on phagocytosis and a phagocytic killing test of C. albicans*

The precipitate from the nonphenolic fraction produced a positive response in both assays. This suggests the active component(s) may consist of amino acids (or peptide), carboxylic acids (or their salts) and/or sugar moiety.

*Examination of amino acid composition in the active precipitate*

From amino acid analysis of the active precipitate, the following composition (%) of amino acids was identified: Asp (9.9), Thr (1.7), Ser (2.4), Glu (8.9), Gly (6.2), Ala (7.1), Cys (8.7), Val (8.7), Met (1.7), Ile (2.0), Leu (6.6), Tyr (9.0), Phe (4.5), Lys (2.0), His (3.8), Orn (3.2), Arg (2.5) and Pro (10.3). Among these amino acids the contents of cysteine and proline were higher than in the active nonphenolic substance.

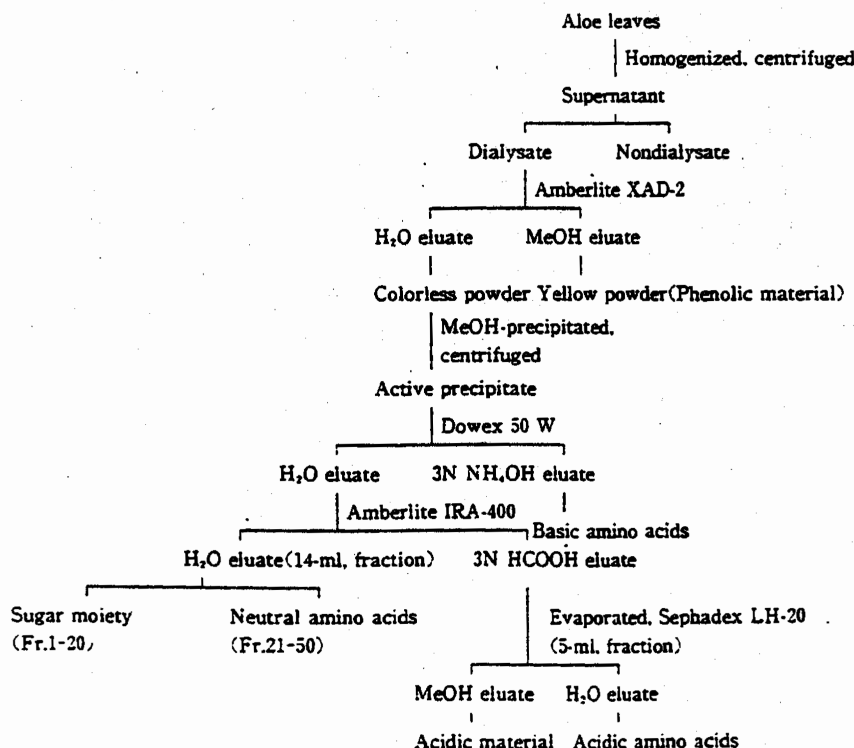


Fig. 1. Separation of neutral, acidic and alkaline materials.

Table 2 Effects of the fraction isolated on phagocytosis and a phagocytic killing test of *Candida albicans*

| | Dilution | Phagocytosis | Dilution | Phagocytic killing test |
|---|---|---|---|---|
| Dialyzed material | 1:1 | 1.021±0.252 | 1 | 0.304±0.093*** |
| | 1:5 | 1.259±0.144 | $10^{-1}$ | 0.761±0.113 |
| | 1:10 | 0.980±0.100 | $10^{-2}$ | 0.923±0.123 |
| | 1:20 | 1.058±0.295 | $10^{-3}$ | 0.964±0.077 |
| | | | $10^{-4}$ | 0.980±0.067 |
| Nonphenolic substance | 1:1 | 1.013±0.027 | 1 | 1.482±0.348 |
| | 1:5 | 1.156±0.095 | $10^{-1}$ | 1.263±0.205 |
| | 1:10 | 1.171±0.074 | $10^{-2}$ | 1.317±0.129 |
| | 1:20 | 1.068±0.055 | $10^{-3}$ | 1.225±0.125 |
| | | | $10^{-4}$ | 1.197±0.161 |
| Phenolic substance | 1:1 | 0.781±0.040 | 1 | 0.690±0.169 |
| | 1:5 | 0.971±0.075 | $10^{-1}$ | 0.928±0.076 |
| | 1:10 | 1.007±0.091 | $10^{-2}$ | 1.099±0.082 |
| | 1:20 | 0.888±0.017 | $10^{-3}$ | 1.092±0.018 |
| | | | $10^{-4}$ | 1.010±0.044 |
| Active precipitate | 1:1 | 1.217±0.089 | 1 | 1.432±0.307 |
| | 1:5 | 1.316±0.070 | $10^{-1}$ | 1.428±0.305 |
| | 1:10 | 1.371±0.145 | $10^{-2}$ | 1.523±0.291 |
| | 1:20 | 1.306±0.095 | $10^{-3}$ | 1.408±0.229 |
| | | | $10^{-4}$ | 1.340±0.193 |
| Neutral amino acids fraction | 1:1 | 0.938±0.020 | 1 | 1.519±0.240* |
| | 1:5 | 0.997±0.031 | $10^{-1}$ | 1.173±0.290 |
| | 1:10 | 1.143±0.038** | $10^{-2}$ | 1.032±0.323 |
| | 1:20 | 1.919±0.088*** | $10^{-3}$ | 1.214±0.072 |
| | | | $10^{-4}$ | 0.786±0.071 |

Each sample (20 mg) was dissolved in 5 ml of 0.15M PBS, pH 7.2 and tested for phagocytosis. The values for phagocytosis are presented as a phagocytic index. The phagocytic index of the control was calculated to be 1.000. Each sample was dissolved in 0.15M PBS, pH 7.2, at concentrations of 1 to $10^{-4}$ mg/ml and tested for phagocytic killing of *C. albicans*. The values for the phagocytic killing test are presented as a phagocytic index of killed *C. albicans*. The phagocytic index of the control was calculated to be 1.000. Data were analyzed using student's t-test to determine the significant difference. The values in these assays are mean±s.e. of 6 samples.

* ** *** : statistically significant from the control at $p<0.01$, 0.02 and 0.05, respectively.

*Examination of neutral, acidic and alkaline fractions*

Chromatographic separation with anion or cation exchange resins was done and neutral, acidic and alkaline fractions were obtained. In the $H_2O$ eluate from chromatography on Amberlite IRA-400, glucose and mannose were identified by TLC and GC. The acidic fraction was separated on a Sephadex LH-20 column to give acids and acidic amino acids. GC on the methyl ester of the acids showed the presence of citric, (+)-isocitric, malic and L (−)-2-hydroxy-4-carboxy-2-hexene-dicarboxylic acids. The activity found in the precipitate, however, disappeared and could not be detected in the sugar moiety, acidic fraction (carboxylic acids and acidic amino acids) or alkaline fraction (Fig. 1). Only the neutral amino acid fraction showed a significant activity for both phagocytosis tests. From these findings it is reasonable to speculate that the active component(s)

Table 3 Effects of cysteine, proline and a mixture of cysteine and proline on phagocytosis and a phagocytic killing test of *Candida albicans*

| | Dilution | Phagocytosis | Dilution | Phagocytic killing test |
|---|---|---|---|---|
| Cysteine | 1:1 | 1.127±0.019* | 1 | 1.290±0.307 |
| | 1:5 | 1.173±0.055 | $10^{-1}$ | 1.106±0.134 |
| | 1:10 | 1.130±0.028** | $10^{-2}$ | 1.635±0.257 |
| | 1:20 | 1.212±0.082 | $10^{-3}$ | 1.601±0.267 |
| | | | $10^{-4}$ | 1.970±0.286** |
| | | | $10^{-5}$ | 1.742±0.178* |
| Proline | 1:1 | 0.920±0.092 | 1 | 1.724±0.386 |
| | 1:5 | 1.080±0.045 | $10^{-1}$ | 1.585±0.207*** |
| | 1:10 | 1.209±0.127 | $10^{-2}$ | 2.068±0.569 |
| | 1:20 | 1.144±0.048 | $10^{-3}$ | 1.661±0.297 |
| | | | $10^{-4}$ | 1.777±0.217** |
| | | | $10^{-5}$ | 1.340±0.315 |
| A mix. of cysteine and proline | 1:1 | 1.137±0.045*** | 1 | 0.566±0.392 |
| | 1:5 | 1.135±0.039** | $10^{-1}$ | 1.104±0.226 |
| | 1:10 | 1.221±0.023* | $10^{-2}$ | 1.501±0.107* |
| | 1:20 | 1.219±0.083*** | $10^{-3}$ | 0.996±0.408 |
| | | | $10^{-4}$ | 1.573±0.237 |
| | | | $10^{-5}$ | 1.441±0.161*** |
| Tuftsin | 1:1 | 0.988±0.059 | 1 | 0.869±0.150 |
| | 1:5 | 1.053±0.053 | $10^{-1}$ | 0.933±0.111 |
| | 1:10 | 1.126±0.072* | $10^{-2}$ | 0.934±0.156 |
| | 1:20 | 1.121±0.054* | $10^{-3}$ | 1.127±0.045 |
| | | | $10^{-4}$ | 1.044±0.028 |
| | | | $10^{-5}$ | 1.103±0.100 |

Each sample (20 mg) was dissolved in 5 ml of 0.15M PBS, pH 7.2 and tested for phagocytosis. The values for phagocytosis are presented as a phagocytic index. The phagocytic index of the control was calculated to be 1.000. Each sample was dissolved in 0.15M PBS, pH 7.2, at concentrations of 1 to $10^{-5}$ mg/ml and tested for phagocytic killing of *C. albicans*. The values for the phagocytic killing test are presented as a phagocytic index of killed *C. albicans*. The phagocytic index of the control was calculated to be 1.000. Data were analyzed using student's t-test to determine the significant difference. The values in these assays are mean±s.e. of 6 samples.

*, **, ***: Statistically significant from the control at $p<0.01$, 0.02 and 0.05, respectively.

might be oxidized during the chromatographic separation on ion exchange resins, and the participation of cysteine and neutral amino acids(s) for phagocytosis was strongly suggested. The following amino acid composition (%) were obtained: in the neutral amino acid fraction, Asp (6.7), Thr (2.9), Ser (4.6), Glu (5.2), Gly (10.0), Ala (33.3), Val (5.5), Ile (1.1), Leu (2.4), Tyr (3.4), Pro (23.9): in the acidic amino acid fraction, Asp (1.0), Thr (1.7), Glu (37.1), Gly (4.7), Ala (30.5), Val (8.2), Phe (4.6), Pro (11.4): in the alkaline fraction, Thr (3.5), Ser (12.1), Gly (15.4), Ala (5.9), Val (3.8), Ile (3.8), Leu (4.8), Phe (3.5), Lys (29.2), His (7.3), Arg (3.8). In the neutral amino acid fraction the contents of alanine and proline were high and the possible participation of these amino acids in the phagocytosis was suggested (Table 2).

*Effect of cysteine, proline and a mixture of cysteine and proline (1:1) on phagocytosis and a phagocytic killing test of C. albicans*

Comparison of the amino acid composition of the active precipitate, neutral, acidic and alkaline fractions suggested a strong participation by cysteine, alanine and proline. Individual amino acids, except cysteine and proline and mixtures of neutral, acidic and alkaline amino acids were inactive, while tuftsin (Thr-Lys-Pro-Arg)[6] significantly stimulated the phagocytosis activity of neutrophils, though a dose-dependent response was not observed. A mixture of cysteine and proline (1:1) showed significant activity in phagocytosis assays (Table 3).

## DISCUSSION

The phagocytic action of neutrophils is strongly dependent on the energy derived from oxidative phosphorylation and glycolysis[7], and substances affecting -SH groups and chelating agents for $Mg^{2+}$ and $Ca^{2+}$ inhibit the phagocytosis of neutrophils[8]. These findings are explained in terms of the contribution of -SH groups to the phagocytic action of neutrophils. Recently, the chemical and biological properties of tuftsin and its analogues have been studied. There was no stimulation of phagocytosis by normal polymorphonuclear leukocytes by tuftsin and single amino acids[9]. However, recent studies on the relationship between the structure and function of tuftsin showed that tuftsin activity is dependent on only two amino acids, proline and arginine[10], and the strong stimulation of phagocytosis of guinea pig neutrophils was noted in the presence of Leu, Ile, Pro, Arg and His[11]. In addition, tuftsin normalized only the impaired phagocytosis of granulocytes and blasts of children and did not show any stimulating effect on normal phagocytes at all[12]. In the present experiment no dose-dependent effect of tuftsin, active fraction and a mixture of cysteine and proline in stimulating phagocytosis was observed. This finding may be explained as a specific binding of cysteine, proline and tuftsin to target cells as was reported in an earlier experiment[6]. Phagocytosis-stimulating effect of the proline or the arginine near the proline residue in tuftsin molecule was reported, suggesting a basic amino acid near the proline residue plays an important role for phagocytosis-stimulation[13]. In our experiment, however, proline together with cysteine showed a significant stimulation for phagocytosis. The present investigation provides a basis for considering the mechanism of phagocytosis stimulation and the role of single amino acids.

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# 成人気管支喘息・末梢血白血球の貪食能に関する アロエ・エキス中のアミノ酸の影響

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キダチアロエ・エキス6カ月間の経口投与実験は，成人慢性気管支喘息患者への有効性を示した．この事実はキダチアロエ・エキスに抗感染作用物質の存在を示唆している．患者白血球の貪食能と nitro-blue tetrazolium chloride 還元能テストを指標とした感染防御物質の検索を行い，多糖類（β-1,4-0-linked acetyl mannan，分子量40000）と糖蛋白（蛋白57%，ヘキソサミン4%，糖類34%，分子量40000）を単離した．これら活性物質の検索中，低分子量の感染防御物質の存在が示唆されたので，今回，患者白血球の貪食能と *Candida* killing test を指標とした活性成分の検索を行った．その結果，アロエ・エキスの透析外液に活性が認められた．そこで，各種イオン交換樹脂やクロマトグラフィーを用いた分画・精製を行ったところ，中性アミノ酸画分に活性が移行したのを認めた．各画分のアミノ酸組成と白血球貪食能亢進活性の比較から，cysteine と proline の関与が示唆された．tuftsin を内部標準物質とした活性の検討から cysteine と proline（1：1）が有意に患者白血球貪食能亢進活性を示した．

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