PURIFICATION AND CHARACTERIZATION OF Β-GLUCANASE FROM GANODERMA TSUGAE

李思樺、陳鴻章

E-mail: 9126584@mail.dyu.edu.tw

ABSTRACT

The bioactive carcinostatic substance in Lingzhi is high molecular weight polysaccharides linked by β-(1,3)-D-glucosidic bond. In this project, the β-glucanases from Ganoderma tsugae were isolated, purified and characterized. Purification of the β-1,3 glucanase from Ganoderma tsugae with a recovery of 4.2% and purity increase of 15.1 fold was achieved by ammonium sulfate fractionation, Sephadex G-50 and DEAE Sepharose CL-6B chromatography. The optimum pH for β-1,3 glucanase was 5.0. The enzyme was stable at pH 6.0. The optimum temperature for β-1,3 glucanase was 50℃. The enzyme was rapidly denatured at temperature of 60℃ and above. Substrate specificity studies indicated this enzyme has maximum activity toward laminarin. The relative rate of hydrolysis of curdlan, lichenan and zymosan to that of laminarin was 16.1, 43.7 and 26.3%, respectively. Almost complete inhibition was observed with 10 mM Cu+2, while moderate inhibition was seen with Fe+2, Zn+2, Mn+2, Mg+2, Na+2及Ca+2. Activity was unaffected by 10 mM Co+2. All the common inhibitors tested inhibited β-1,3 glucanase activity to various extents. The enzyme was completely inhibited by 10 mM L-asccorbic acid. The Km value of β-1,3 glucanase toward laminarin was 5.99 mg/ml, while Vmax was 129.87 OD/min. ml. Purification of the β-1,6 glucanase from Ganoderma tsugae with a recovery of 3.2% and purity increase of 15.6 fold was achieved by ammonium sulfate fractionation, Sephadex G-50 and DEAE Sepharose CL-6B chromatography. β-1,6 glucanase displayed relatively broad pH optimum. The enzyme was stable at pH 6.0. The optimum temperature for β-1,6 glucanase was 60℃. The enzyme was rapidly denatured at temperature of 50℃ and above. Substrate specificity studies indicated this enzyme has maximum activity toward pustulan. The relative rate of hydrolysis of zymosan to that of pustulan was 48.3%. Almost complete inhibition was observed with 10 mM Ca+2, while moderate inhibition was seen with Fe+2, Zn+2, Mn+2, Mg+2, Na+2及Cu+2. Activity was unaffected by 10 mM Co+2. All the common inhibitors tested inhibited β-1,6 glucanase activity to various extents. The enzyme was completely inhibited by 10 mM Sodium metabisulfite. The Km value of β-1,6 glucanase of toward pustulan was 1.88 mg/ml, while Vmax was 75.19 OD/min. ml. This study displayed that β-1,3 glucanase and β-1,6 glucanase from Ganoderma tsugae only hydrolyze specific 1→3 and 1→6 linkage types of β-glucans, respectively. Therefore, substrate specific β-1,3 glucanase and β-1,6 glucanase could be employed in the enzymatic assay to identify the authenticity of Lingzhi products on the market.

Keywords : Ganoderma tsugae ; bioactive ; polysaccharide ; β-glucanase ; enzymatic assay

Table of Contents

REFERENCES

1.丁懷謙（2000）。食藥用菇多醣體之免疫生理活性。食品工業32（5） 28-42。 2.王伯徹、邱世浩、黃仁彰（1998）。食用菇保健食品特輯。食品工業30（5）1-36。 3.王伯徹、林錦堂、華傑（1991）。靈芝菌種之長期保存。中國農業化學會誌28（1）86-93。 4.水野 卓及川合正允（1992）。菇類的化學生化學。國立編譯館。台北。 5.唐瑞菁、程梅萍（1992）。靈芝培養基的探討-酵母抽出物的取代。國立雲林技術學院學報4（1） 145-156。 6.陳國城（1999）。微生物酵素工程。P.33。 7.梁志欽（1992）。松杉靈芝浸漬培養產生的1,3-BETA-GLUCANASE對菌體外多醣的影響。國立台灣大 學農業化學研究所碩士論文。 8.莊榮輝、蘇仲卿（1986）。蛋白質膠體電泳檢定法。P.69-85。 9.許瑞祥（1990）。靈芝屬菌株鑑定系統之研究。國立台灣大學農業化學研究所博士論文。 10.許瑞祥（1995）。靈芝的研究現況與展望。生物產業 （BIOINDUSTRY），6（4），289-296。 11.曾浩洋 (1996)。飼料用酵素及乳酸菌之生化評估及相關乳酸菌之分子分類。行政院國科會 PB8211 -2080。 12.傅偉光（1999）。靈芝中機能性成分分析方法之簡介。健康靈芝（冬季號），15-18。 13.趙東旭、楊新林、王幫武、朱鶴孫 （1999）靈芝研究的若干進展。食用菌學報6（3）59-64。 14.蘇遠志、黃世佑 (1994)。微生物化學工程。P.594。 15.AINSWORTH, G. C., SPARROW, F. K., AND SUSSMAN, A. S. (1973). THE FUNGI. VOL. IVA. A TAX ONOMIC REVIEW WITH KEY: ASCOMYCETES AND FUNGI IMPERFECTI. ACADEMIC PRESS, INC. NEW YORK .621PP. 16.AUTIO, K., SIMOINEN, T., SUORTTI, T., SALMENKALLIO, M. M., LASSILA, K.AND WILHELMSON,A. (2001). STRUCTURAL AND ENZYMIC CHANGES IN GERMINATED BARLEY AND RYE.JOURNAL-OF-THE-INST ITUTE-OF-BREWING, 107 (1) 19-25, 32 REF. 17.BARRAS, D. R.(1972) A GLUCAN ENDO-HYDROLASE FROM SCHIZOSACCHAR- OMYCES POMBE AND ITS RO -LE IN CELL WALL GROWTH. ANTON. LEEUWEN, 38(1):65-80. 18.BENGTSSON, S. (1992) STUDIES ON STRUCTURES AND PROPERTIES OF SOLUBLE CELL-WALL POLYSACC HARIDES IN RYE AND BARLEY. DISSERTATION-ABSTRACTS NTERNATIONAL, 53 (4) 649. 19.BRADFORD, M. (1976) A RAPID AND SENSITIVE METHOD FOR THE QUANTITATION OF MICROGRAM QUAN -TITIES OF PROTEIN UTILIZING THE PRINCIPLE OF PROTEIN-DYE BINDING. ANALYTICAL BIOCHEMIS -TRY, 72:248-254. 20.BODENMANN, J., HEINIGER, U. AND HOHL, H. R. (1985) EXTRACELLULAR ENZYME OF PHYTOPHTHORA INFESTANS:ENDO-CELLULASE, BETA- GLUCOSIDASES AND 1,3-BETA-GLUCANASE. CAN. J. MICROBIOL, 31(1)75-81. 21.CAI, Y. J., BUSWELL, J. A., AND CHANG, S. T. (1998). B -GLUCANASE COMPONENTS OF THE CEL -LULOLYTIC SYSTEM OF THE EDIBLE STRAW MUSHROOM, VOLVARIELLA VOLVACEA. ENZYME AND MICR -OBIAL TECHNOLOGY, 22:122-129. 22.CARDER, J. H. (1986) DETECTION AND QUANTIFICATION OF CELLULASE BY CONGO RED STAINING OF SUBSTRATES IN A CUP-PLATE DIFFUSION ASSAY. ANAL. BIOCHEM, 153:75-82. 23.CHANG, R. (1994) EFFECTIVE DOSE OF GANODERMA IN HUMANS. IN PROC. CONTRIBUTED SYMPOSIUM 59A,B. 5TH INTL. MYCOL. CONGR., BUCHANAN PK, HSEU RS AND MONCALVO JM(EDS), TAIPEI,P.101 -13. 24.CHANG, R. (1996) THE CENTRAL IMPORTANCE OF THE 7-GLUCAN RECEPTOR AS THE BASIS OF IMMUNO -LOGIC BIOACTIVITY OF GANODERMA POLYSACCHARIDE. IN REISHI, MIZUNO T, KIM BK(EDS),II YAN -G PRESS, SEOUL, P.153-159. 25.CHASTERS, C. G. C., AND BULL. A. T.(1963) THE ENZYMIC DEGRADATION OF LAMINARIN. 1. THE DISTRIBUTION OF LAMINARINASE AMONG MICROORGANISMS. BIOCHEM. J, 86:28-31. 26.CHASTERS, C. G. C., AND BULL. A. T.(1963) THE ENZYMIC DEGRADATION OF LAMINARIN.. 2.THE MULTICOMPONENT NATURE OF FUNGAL LAMINARINASES. BIOCHEM. J, 86:31-38. 27.CHASTERS, C. G. C., AND BULL. A. T.(1963) THE ENZYMIC DEGRADATION OF LAMINARIN.3. SOME EFFECTS OF TEMPERATURE, PH AND VARIOUS CHEMICAL REAGENTS ON FUNGAL LAMINARINASES.BIOCH -EM. J, 86:38-45. 28.DAVIS, B. J. (1964) DISC ELECTROPHORESIS. METHOD AND APPLICATION TO HUMAN SERUM PROTEI N. ANN. N. Y. ACAD. SCI, 212(2):404-427. 29.DILL, I AND G. KRAEPELIN. (1986) MODEL FOR EXTENSIVE DELIGNIFICATION OF WOOD BY GANODE -RMA APPLANATUM. APPLIED-AND-ENVIRONMENT- MICROBIOLOGY, 52: 1305-1312. 30.DINGLE, J., REID, W. W. AND SOLOMONS, E. L. (1953) THE ENZYMATIC DEGRADATION OF PECTIN AND OTHER POLYSACCHARIDES. APPLICATION OF THE CUP PLATE ASSAY TO THE ESTIMATION OF EN -ZYME. J. SCI. FOOD. AGRIC, 4:149-154. 31.DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A. AND SMITH, F. (1956).COLORIM -ETRIC METHOD FOR DETERMINATION OF SUGARS AND RELATED SUBSTANCES. ANAL. CHEM, 28: 350- 356. 32.DOBOURDIEU, D., DESPLANQUES, C., VILLETTAZ, J. AND RIBEREAU-GAYON, P. (1985) INVESTIGA -TIONS OF AN INDUSTRIAL BETA-D-GLUCANASE FROM TRICHODERMA HARZIANUM. CARBOHYDR. RES, 144(2): 277-287. 33.ELWINGER, K. AND SATERBY, B. (1987) THE USE OF BETA-GLUCANASE IN PRACTICAL BROILER DIE -TS CONTAINING BARLY OR OAT. SWED. J. AGRIC. RES, 17:133-137. 34.FLEET, G. H. AND PHAFF, H. (1981) IN ENCYCLOPEDIA OF PLANT PHYSIOLOGY 13B (TANNER, W. AND LOEWOUS, F. A., EDS) SPRINGER-VERLAG, BERLIN, PP. 416-440. 35.FLEET, G. H. AND PHAFF, H. (1975) GLUCANASE IN SCHIZOSACCHAROMYCES. ISOLATION AND PROP -ERTIES OF AN EXO-BETA-GLUCANASE FROM THE CELL EXTRACT AND CULTURE FLUID OF SCHIZOSACC -HAROMYCES JAPONICUS VAR. VERSATILIS. BIOCHEM. BIOPHYS. ACTA, 410(2):318-332. 36.FLEET, G. H. AND PHAFF, H. (1974) GLUCANASE IN SCHIZOSACCHAROMYCES. ISOLATION AND PROP -ERTIES OF THE CELL WALL-ASSOCIATED BETA-1,3- GLUCANASE. J. BIOL. CHEM, 249:1717-1728. 37.FISKE, M. J., TOBEY-FINCHER, K. L. AND FUCHS, R. L. (1990) CLONING OF TWO GENES FROM B -ACILLUS CIRCULANS WL-12 WHICH ENCODE 1,3-BETA-GLUCANASE ACTIVITY. J. GEN. MICROBIOL, 136(12): 2377-2383. 38.HIEN, N. H. AND FLEET, G. H. (1983) SEPARATION AND CHARCTERIZATION OF SIX B -(1→3)-GL -UCANASE FROM SACCHAROMYCES CEREVISIAE. J. BACTERIAL, 156:1204-1213. 39.KARINEN, P. AND LEHTOMAEKI, I. (1990) PROCEDURE FOR PRODUCING FINE FIBRE, AND F -INE FIBRE.PCT-INTERNATIONAL-PATENT-APPLICATION; WO 90/10392 A1, FI 89-1110

(19890308) [ALKO, SF-00101 HELSINKI, FINLAND]. 40.KOBAYASHI, R., MIWA, T., YAMAMOTO, S. AND NAGASAKI, S. (1980) PURIFICATION AND CHARACT -ERIZATION OF A YEAST CELL-LYTIC ENZYME OF A SPECIES OF RHIZOCTONIA. J. FERMENT. TECHN -OL, 58(3):319-326. 41.KOBAYASHI, R., MIWA, T., YAMAMOTO, S. AND NAGASAKI, S. (1981) PROPERTIES AND MODE OF AC -TION OF BETA-1,3-GLUCANASE FROM RHIZOCTONIA SP.
[FUNGI]. J. FERMENT. TECHNOL, 59(1):21 -26. 42.KUSAMA, S., KUSAKABE, I. AND MURAKAMI, K. (1986) PURIFICATION AND SOME PROPERTIES OF DE -TA-1,3-GLUCANASE FROM STREPTOMYCES SP. AGRIC. BIO. CHEM, 50(5): 1101-1106. 43.LAURENT, T. C. AND KILLANDER, J. (1964) A THEORY OF GEL FILTRATION AND ITS EXPERIMENTAL VERIFICATION. J. CHROMATOGR, 14:317-330. 44.LEE, C. J., HORSLEY, R. D., MANTHEY, F. A. AND SCHWARZ, P. B. (1997) COMPARISONS OF B -GLUCAN CONTENT OF BARLEY AND OAT. CEREAL CHEM, 74(5): 571-575. 45.I. U. B. (1984) ENZYME NOMENCLATURE; RECOMMENDATIONS OF THE NOMENCLATURE COMMITTEE OF THE INTERNATIONAL UNION OF BIOCHEMISTRY. ACADEMIC PRESS, ORLANDO. 46.MARTIN, D. F., PRIEST, F. G., TODD, C. AND GOODFELLOW, M. (1980) DISTRIBUTION OF BETA -GLUCANASES WHITIN THE GENUS BACILLUS. APPL. ENVIRON. MICROBIOL, 40(6): 1136-1138. 47.MCCLEARY, B. V. (2000) IMPORTANCE OF ENZYME PURITY AND ACTIVITY IN THE MEASUREME -NT OF TOTAL DIETARY FIBER AND DIETARY FIBER COMPONENTS. JOURNAL-OF-AOAC-INTERNATIONAL, 83 (4): 997-1005. 48.MERATAD, O. AND MCNAB, J. M. (1975) THE EFFECT OF HEAT TREATMENT AND ENZYME SUPPLEMENT -ATION ON THE NUTRITIVE VALUE OF BARLEY FOR BROILER CHICKS. BR. POULT, 16(1): 68-72. 49.MOSS, B. R., BEECHLER, C. W., NEWMAN, C. W. AND EI-NEGOUMY, A. M. (1977) ENZYME SUPPLE -MENTATION OF BROILERS RATIONS. POULT. SCI, 56:1741-1745. 50.NOTARIO, V. (1982) BETA-GLUCANASES FROM CANDIDA ALBICANS : PURIFICATION, CHARACTERIZAT -ION AND THE NATURE OF THEIR ATTACHMENT TO CELL WALL COMPONENT [FUNGI]. J. GEN. MICROB -IOL, 128:747-759. 51.NOTARIO, V., VILLA, T. G., BENITEZ, T. AND VILLANUEVA, J. R. (1976) BETA-GLUCANASE IN THE YEAST CRYPTOCOCCUS ALBIDUS VAR. AERIUS. PRODUCTION AND SEPARATION OF BETA-GLUCANAS -ES IN ASYNCHRONOLDS CULTURE. CAN. J. MICROBIOL, 22(2):261-268. 52.PETERSEN, B. O., KRAH, M., DUUS, J. O. AND THOMSEN, K. K. (2000) A TRANSGLYCOSYLATING 1,3(4)-BETA-GLUCANASE FROM RHODOTHERMUS MARINUS. NMR ANALYSIS OF ENZYME REACTIONS. EUR -OPEAN-JOURNAL- -BIOCHEMISTRY, 267 (2) : 361-369. 53.PETTERSSON, D. AND AMAN, P. (1989) ENZYME SUPPLEMENTATION OF A POULTRY DIET CONTAINING RYE AND WHEAT. BR. J. NUTR, 62:139-146. 54.PITSON, S. M., SEVIOUR, R. J. AND MCDOUGALL, B. M. (1997) PURIFICATION AND CHARACTERIZ -ATION OF AN EXTRACELLULAR BETA -GLUCOSIDASE FROM THE FILAMENTOUS FUNGUS ACREMONIUM PE -RSICINUM AND ITS PROBABLE ROLE IN BETA -GLUCAN DEGRADATION. ENZYME AND MICROBIAL TECH -NOLOGY, 21:182-190. 55.PITSON, S. M., SEVIOUR, R. J., AND MCDOUGALL, B. M. (1993) NONCELLULOLYTIC FUNGAL BETA -GLUCANASES: THEIR PHYSIOLOGY AND REGULATION. ENZYME- AND-MICROBIAL-TECHNOLOGY, 15 (3) :178-192. 56.PITSON, S. M., SEVIOUR, R. J. AND MCDOUGALL, B. M. (1996) PROTEOLYTIC INACTIVATION OF AN EXTRACELLULAR (1,3)-BETA-GLUCANASE FROM THE FUNGUS ACREMONIUM PERSICINUM IS ASSOCIA -TED WITH GROWTH AT NEUTRAL OR ALKALINE MEDIUM PH. FEMS-MICROBIOLOGY-LETTERS, 145 (2) 287-293. 57.REESE, E. T. (1977) RECENT ADVANCES IN PHYTOCHEMISTRY 11 (LOEWUS, F. A. AND RUNECKLES, V. C., EDS) PLENUM, NEW YORK, PP. 311-367. 58.REYES, F. AND LAHOZ, R (1977) VARIATION IN LYSIS OF WALLS OF SCLEROTINIA FRUCTIGENA WI -TH AGE OF CULTURE. J. GEN. MICROCIOL, 98:607-610. 59.RIDOUT, C. J., COLEY-SMITH, J. R. AND LYNCH, J. M. (1986) ENZYME ACTIVITY ELECTROPHEOR -ETIC PROFILE OF EXTRACELLULAR PROTEIN INDUCED IN TRICHODERMA SPP. BY CELL WALLS OF RH -IZOCTONIA SOLANI. J. GEN. MICROBIOL, 132(8):2345-2352. 60.SANCHEZ, M., NOMBELA, C., VILLANUEVA, J. R. AND SANTOS, T (1982) PURIFICATION AND PART -IAL CHARACTERIZATION OF A DEVELOPMENTALLY REGULATED 1,3-BETA-GLUCANASE FROM PENCILLI -UM ITALICUM [FUNGI]. J. GEN. MICROBIOL, 128:2047-2053. 61.SANTOS, T., VILLANUEVA., J. R. AND NOMBELA, C. (1977) PRODUCTION AND CATABOLITE REPRES -SION OF PENICILLIUM ITALICUM BETA-GLUCANASES. J. BACTERIOL, 129(1):52-58. 62.SANTOS, T., VILLANUEVA., J. R. AND NOMBELA, C. (1978) REGULATION OF BETA-1,3-GLUCANASE SYNTHESIS PENICILLIUM ITALICUM. J. BACTERIOL, 133(2):542-548. 63.SHINGO, N. AND TAKAHISA, H. (1993) PURIFICATION AND PROPERTIES OF AN EXTRACELLULAR END O-1,4-BETA-GLUCANASE FROM SUSPENSION- CULTURED POPLAR CELLS. PLANT CELL PHYSICAL,34(7) : 1009-1013. 64.SHINSHI, H. AND KATO, K. (1983) PHYSICAL AND CHEMICAL PROPERTIES OF BETA-1,3-GLUCANASE FROM CULTURED TOBACCO CELLS [NICOTIANA TABACUM]. AGRIC. BIOL. CHEM, 47(7):1455-1460. 65.SMITH, D. (1982) LIQUID NITROGEN STORAGE OF FUNGI.TRANS. BR. MYCOL. SOC, 79: 415-421. 66.SMITH, D.(1984). MAINTENANCE OF FUNGI. IN B.E KIRSOP AND J. J. S. SNELL (EDS.), MAINTE -NANCE OF MICROORGANISM: A MANUAL OF LABORATORY METHODS. ACADEMIC PRESS, LONDON. PP. 83-108. 67.SONE, Y., R. OKUDA, N. WADA, E. KISHIDA, AND A. MISAKI. (1985). STRUCTURE AND ANTITUM -OR ACTIVITIES OF POLYSACCHARIDES ISOLATED FROM FRUITING BODY AND GROWING CULTURE OF MY CELIUM OF GANODERMA ALUCIDIUM. AGRIC. BIOL. CHEM, 49:2641-2653. 68.SOUTH, J. B. (1997) ESTIMATION OF ENDO BETA-GLUCANASE ACTIVITY IN MALT USING A VISCOME -TRIC METHOD. JOURNAL-OF-THE-INSTITUTE-OF-BREWING, 103 (1):3-6. 70.STASINOPOULOS, S. J., AND R. J. SEVIOUR. (1989).

EXOPOLYSACCHSRIDE FORMATION BY ISOLAT -ES OF CEPHALOSPORIUM AND ACREMONIUM. CHEM. PHARM. BULL, 39(3):798-800. 71.TAKASHI AKIYAMA, HANAE KAKU, NAOTO SHIBUYA. (1998). PURIFICATION, CHARACTERIZATION AND NH2-TERMINAL SEQUENCING OF AN ENDO-(1→3, 1→4)- B -GLUCANASE FROM RICE(ORYZA ATIVA)BRA -N. PLANT SCIENCE, 134: 3-10. 72.TOTSUKA, A. AND USUI, T. (1986) SEPARATION AND CHARACTERIZATION OF THE ENDO-BETA-(1 TO 3)-D-GLUCANASE FROM RHIZOCTIONIA SOLANI. AGRIC. BIOL.CHEM, 50(3):543-550. 73.TURNER, P. D. (1965). INFECTION OF OIL PALMS BY GANODERMA. PHYTOPATHOLOGY, 55:937-943. 74.VILLA, T. G., NOTARIO, V., BENITEZ, T. AND VILLANUEVA, J. R. (1976) PURIFICATION OF AN EXO-1,3-BETA-GLUCANASE FROM CANDIDA UTILIS. CAN. J. BIOCHEM, 54(1):927-934. 75.WALSH, G. A., POWER, R. F. AND HEADON, D. R. (1993) ENZYMES IN THE ANIMAL FEED INDUSTRY .TRENDS IN BIOTECHNOL, 11:424-428. 76.WALSH, G. A., MURPHY, R. A., KILLEEN, G. F., HEADON, D. R. AND POWER, R. F. (1995) TECH -NICAL NOTE: DETECTION AND QUANTIFICATION OF SUPPLEMENTATION FUNGAL B -GLUCANASE ACTIV -ITY IN ANIMAL FEED. J. ANIM. SCI, 73:1074-1076. 77.WANG G., ZHANG J., MIZUNO T., ZHUANG C., ITO H., MAYUZUMI H.,OKAMOTO H. AND LI J.(1993) .ANTITUMOR ACTIVE POLYSACCHARIDES FROM THE CHINESE MUSHROOM SONG SHAN LINGZHI, THE FRU -ITING BODY OF GANODERMA TSUGAE. BIOSCI. BIOTECH. BIOCHEM, 57(6):894-900. 78.WOOD, P. J.(1994) EVALUATION OF OAT BRAN AS A SOLUBLE FIBER SOURCE. CHARACTERIZATION OF OAT B -GLUCAN AND ITS EFFECTS ON GLYCERNIC RESPONSE. CARBOHYDRATE POLYMERS, 25:331-336. 79.WOOD, P. J. (1981) THE USE OF DYE-POLYSACCHARIDE INTERACTIONS IN BETA-D-GLUCANASE ASSAY .CARBOHYDR. RES, 94:C19. 80.YAMAMOTO, R. AND NEVINS, D. J. (1983) DEGRADATION OF A GLUCAN CONTAINING BETA-(1 TO 3) AND BETA-(1 TO 6) LINKAGES BY EXO-(1 TO 3)-BETA-D-GLUCANASE [ISOLATED FROM A JAPANESE MARINE ALGA KNOWN AS "ARAME", EISENIA BICYCLIS]. CARBOHYDR. RES, 122(2):217-226.