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Characterization of a Novel Carbohydrase from *Lipomyces starkeyi* KSM 22 for Dental Application

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Abstract The combined activities of dextranase and amylase (DXAMase) from *Lipomyces starkeyi* KSM 22 produced from starch fermentation inhibited or prevented dental plaque formation. The activities were stable in commercial mouthwash products; DXAMase activity retained over 93% of original activity after 6 months at 23°C. We examined the effects of enzyme inhibitors and active ingredients in mouthwash on DXAMase activity. The DXAMase was stable with 0.29% (w/v) EDTA, 20% (v/v) ethanol, 0.05% (w/v) fluoride, and 0.05% (w/v) SDS. Among the active ingredients of mouthwash, sodium benzoate (up to 1%, w/v) had no inhibitory effect on either dextranase or amylase activity. In the case of cetylpyridinium chloride, the addition of 0.05% (w/v) inhibited 6% of dextranase activity and 13% of amylase activity. Propylene glycol (up to 1%, w/v) showed no inhibitory effect on either enzyme activity. DXAMase (5 IU/ml) in mouthwash could remove pre-formed films of glucan-bound *S. mutans* cells. The addition of 0.1 IU/ml DXAMase in mouthwash prevented the formation of insoluble-glucan. These *in vitro* properties of *L. starkeyi* KSM 22 DXAMase are desirable for its application as a dental plaque control agent.

Key words: *Lipomyces starkeyi*, dental plaque, mouthwash, carbohydrase

Lipomyces starkeyi, an ascosporogenous yeast, produces an endo-dextranase (EC 3.2.1.11), an enzyme that cleaves the α-1,6-D-glucopyranosyl linkages in dextran using dextran and α-amylase (EC 3.2.1.1) [9]. The yeast has been used in food-related applications, but is not known to produce antibiotics nor toxic metabolites [21]. *L. starkeyi* dextranase has been used to treat successfully a dextran-contaminated sugar process [12]. Except for a few bacterial dextranases, microbial dextranases generally are inducible [1, 13]. Kim and Day [10] reported on the isolation of both dextranase and amylase from a derepressed and partially constitutive mutant, *L. starkeyi* ATCC 74054, and the characterizations of its enzyme. The mutant was used for the production of small-size dextran, sucrose, and/or starch [9-11]. Dental plaque, the bacterial films adherent to tooth surfaces, is composed of closely packed bacteria and non-cellular material [20]. The major polysaccharide component of some dental plaque is a water-insoluble glucan or 'mutan'. It contributes about 20% of the dry weight of dental plaque. The formation of this sticky polymer appears to be one of the major determinants of cariogenicity [2, 4, 15, 22]. Structural studies of the extracellular glucans produced from *Streptococcus mutans* have shown that they exhibit mainly α-1,3-, α-1,4-, and α-1,6-D-glucosidic linkages [6, 24]. Thus, for the efficient removal of dental plaque, mutanolytic, amylolytic, and dextranolytic activities are required. The present report describes the characterizations of the combined activities of dextranase and amylase (DXAMase) prepared from *L. starkeyi* KSM 22, a constitutive and DXAMase hyper-producing mutant, using starch, and its potential use as a dental plaque control agent.

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Materials and Methods

Organisms and Growth Conditions

L. starkeyi KSM 22 was maintained on slants of LW medium containing 1% (w/v) soluble starch and 0.05% (w/v) 2-deoxy-D-glucose. LW medium consists of 0.3% (w/v) yeast extract and 0.3% (w/v) KH_2PO_4. The pH of this medium was adjusted to 4.5 with HCl [9].

Glucan Preparation

Insoluble-glucan was prepared using glucosyltransferase of *Streptococcus mutans*. The strain was grown in 4 liter stirred batch cultures with pH maintained at 7.0. The culture medium

was LW containing 2% (w/v) glucose. After fermentation, the culture was harvested and centrifuged (8,000×g, 15 min). The supernatant (1 l) was mixed with 1 l of sucrose (200 mM) dissolved in 20 mM phosphate buffer (pH 7.0). After complete use of sucrose, any insoluble material was collected by centrifugation (8,000×g, 15 min), washed with water (3 times), and titrated with acetone and ethanol.

Enzyme Purification

L. starkeyi KSM 22 DXAMase was produced in a 4-l Fermentor (Bok-Sung Co., Seoul, Korea) in 3 l of LW medium containing 1% (w/v) starch. The pH was maintained at 4.0 by the addition of 3.0 M NaOH. The aeration rate, temperature, and stirring rate were 1.0 vvm, 30°C, and 100 rpm, respectively. Inocula were 1.5% (v/v) cultures grown for 48 h in LW medium containing 1% (w/v) starch. Culture supernatant was concentrated from 3 l to 200 ml by 70% (w/v) ammonium sulfate precipitation. The concentrate was further concentrated to 50 ml with a Centricon-30 (Amicon). A Carboxymethyl-Sepharose column (75 cm×1.5 cm) was prepared and equilibrated with 20 mM potassium phosphate buffer (pH 6.0). The Centricon-30 concentrate (1.5 ml–20 mg protein/ml) was applied to the column and then eluted with potassium phosphate buffer containing 0.5 M NaCl. The active fractions were pooled and concentrated by isopropanol precipitation. DXAMase fractions from the CM-Sepharose column were size fractionated by Gel Permeation Chromatography on a BIO-RAD A-0.5 m column (70 cm× 2.6 cm) which was prepared and equilibrated with 50 mM citrate phosphate buffer (pH 5.5). Three ml of the CM-Sepharose concentrate fraction was applied to this column (4 mg protein/ml). The fractions with activity were pooled.

Gel Electrophoresis

Analytical slab gel electrophoresis was performed using the technique described by Laemmli [16].

Enzymatic Hydrolysis of Glucans

Glucans were dissolved or suspended in citrate-phosphate buffer (20 mM, pH 5.5) with concentrations of 10 mg/ml for the soluble dextran and 5 mg/ml for the insoluble glucans. 5 IU of enzyme was mixed with 1 ml of the glucans at 37°C. One unit of dextranase or amylase was defined as the amount of enzyme which liberated 1 mole of isomaltose or maltose equivalents in one min using 2% (w/v) dextran or soluble starch as the enzyme substrate, respectively. End products were determined from 48 h hydrolysis reactions. Samples were then analyzed by Thin-layer chromatography as described previously [19].

Effect of DXAMase in Mouthwash Product on the Formation of Insoluble Glucan

To determine whether DXAMase in mouthwash could prevent the formation of films of adherent bacteria, adhesion reactions were allowed to proceed in the presence of sucrose (50 mg/ml) or sucrose plus DXAMase (0.1 IU/ml dextranase activity). Liquid and non-adherent cells were carefully removed by aspiration. Adherent cells were washed once gently with 6.0 ml of 20 mM phosphate buffer, pH 5.8, and then dislodged into 6.0 ml of same buffer by vortex-mixing and dispersed by mild sonication. Adherent biomass was measured by turbidity (A_{550}).

Effect of DXAMase in Mouthwash Product on the Reduction of Pre-Formed Insoluble Glucan

To determine whether DXAMase in mouthwash could remove pre-formed films of adherent bacteria, adhesion reactions were allowed to proceed in the presence of sucrose (50 mg/ml). Liquid and non-adherent cells were carefully removed by aspiration, and adherent cells were washed once gently with 6.0 ml of 20 mM phosphate buffer, pH 5.8. *L. starkeyi* KSM 22 DXAMase (5 IU/ml of dextranase activity in mouthwash) was added and incubation continued for another 24 h at 37°C without shaking. Liquid and non-adherent cells were removed and adherent biomass was washed and measured (A_{550}).

Effects of Enzyme Inhibitors and Chemical Ingredients of Mouthwash on DXAMase Activity

Various kinds and amounts of enzyme inhibitors and chemical ingredients of mouthwash were added into the standard assay buffer to test their effects on DXAMase activity. Suitably diluted test solution was added to the enzyme solution and incubated for 5 min at 37°C, after which time 1 volume of dextran or starch solution, preincubated at 37°C, was added. Activity was determined by the copper-bicinchoninate reducing-value method using 2% substrates [4].

Stability of DXAMase Activity on Commercial Mouthwashes

The stabilities of the DXAMase were tested with four kinds of mouthwash products; A (Johnson & Johnson, U.S.A.), B (Hanmi Pharmaceuticals, Korea), C (Donga Pharmaceuticals, Korea), and D (Ildong Pharmaceuticals, Korea). The remaining enzyme activity after 6 months at 23°C was determined as described above.

RESULTS

Under our enzyme purification condition, we purified a novel carbohydrase from *L. starkeyi* KSM 22 (a 94 K protein on non-denatured SDS-PAGE, 10%) containing both dextranase and amylase activities. We named the protein DXAMase and used it for the following biochemical studies.

Comparison of the Insoluble Glucan Hydrolysis Product from *L. starkeyi* KSM 22 DXAMase and *P. funicoulosum* Dextranase

TLC analysis revealed that the DXAMase produced different hydrolysis products from the insoluble glucan (or mutan) prepared using *S. mutans* glucosyltransferase than did *Penicillium* dextranase (Sigma Chemical Co.). DXAMase produced mostly a glucose but *Penicillium* dextranase produced glucose, isomaltose, and branched-oligosaccharide in small amounts (Fig. 1).

Effect of Enzyme Inhibitors on DXAMase Activity

As shown in Table 1, 0.29% (w/v) EDTA and ethanol (up to 20%, v/v) had no effects on either enzyme activity.

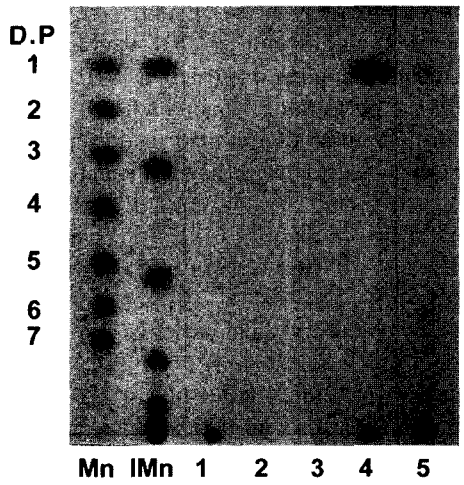

Fig. 1. TLC of insoluble glucan hydrolysis products from *Lipomyces starkeyi* KSM 22 DXAMase and *Penicillium funicoulosum* dextranase.

Mn indicates a series of maltodextrins; IMn indicates a series of isomaltodextrins; Lane 1, *L. starkeyi* KSM 22 DXAMase; Lane 2, *P. funiculosum* dextranase; Lane 3, insoluble glucan prepared using *S. mutans* glucosyltransferase; Lane 4, the hydrolyzate of insoluble glucan by *L. starkeyi* KSM 22 DXAMase; Lane 5, the hydrolyzate of insoluble glucan by *P. funiculosum* dextranase. D.P: degree of polymerization.

Table 1. Inhibition of various chemicals on the dextranase and amylase activities of *Lipomyces starkeyi* KSM 22 DXAMase.

Inhibitor	Concentration (%)	Relative activity (%)	
		Dextranase	Amylase
Control	-	100	100
EDTA	0.29	100	100
SDS	0.05	91	92
	0.5	85	68
Fluoride	0.05	94	91
Ethanol	1	99	100
	5	98	98
	10	98	97
	20	97	97

Details of the reaction condition are described in Materials and Methods.

Fluoride at 0.05% (w/v) and 0.05% (w/v) SDS still showed over 90% of both enzyme activities.

Stability of DXAMase on Commercial Mouthwashes

We have tested the stability of DXAMase on four commercial mouthwash products for extended periods. Except for mouthwash product C, both dextranase and amylase activities were stable in the other three commercial products (over 93% of original activity left) for longer than 6 months at 23°C (Fig. 2).

Inactivation Effect of Chemical Ingredients of Mouthwash on *L. starkeyi* KSM 22 DXAMase Activity

Sodium benzoate and propylene glycol had no inactivation effect on either dextranase and amylase activity of KSM 22 DXAMase. In the case of cetylpyridium chloride, the inactivation rate was concentration dependent. At 0.05% (w/v), which is the concentration in mouthwash, dextranase activity was reduced to 6% and amylase activity was reduced to 13%.

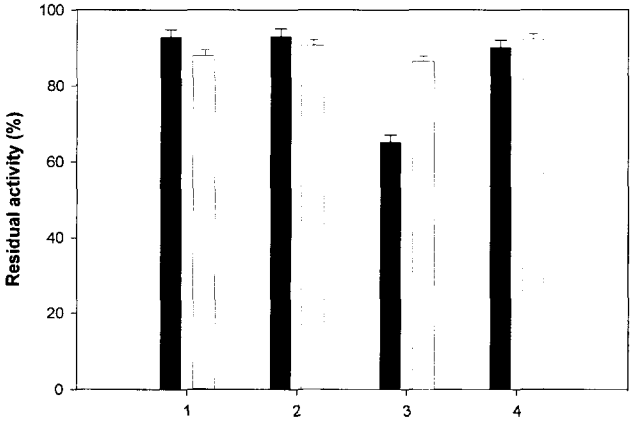

Fig. 2. Stabilities of DXAMase activity on commercial mouthwashes.

1, mouthwash product A; 2, mouthwash product B; 3, mouthwash product C; 4, mouthwash product D. Dextranase activity (■); amylase activity (□).

Table 2. Effect of chemical ingredients of mouthwash on *Lipomyces starkeyi* KSM 22 dextranase and amylase activities.

Chemical ingredients	Concentration (%)	Relative activity (%)	
		Dextranase	Amylase
Control		100	100
Sodium benzoate	1	100	100
Propylene glycol	1	100	100
Cetylpyridinium chloride	0.001	100	100
	0.01	99	95
	0.05	94	87
	0.1	88	80
	0.5	71	62
	1	67	56

Details of the reaction condition are described in Materials and Methods.

Fig. 3. Effect of *Lipomyces starkeyi* KSM 22 DXAMase in mouthwash product A on the formation of insoluble glucan.
DXAMase was incubated in the reaction digest: 1, sucrose + glucosyltransferase; 2, sucrose + glucosyltransferase + mouthwash product A; 3, sucrose + glucosyltransferase + DXAMase in mouthwash product A.

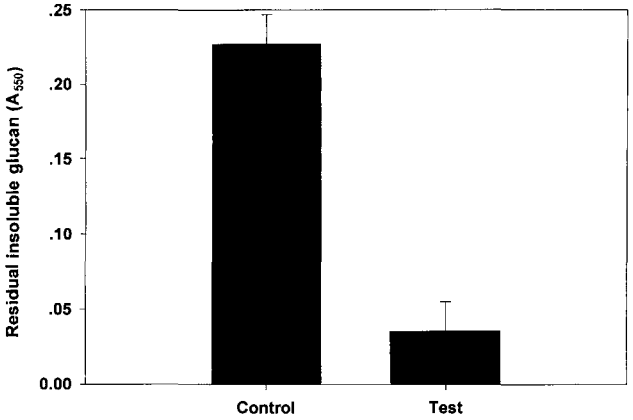

Fig. 4. Reduction of pre-formed insoluble glucan by *Lipomyces starkeyi* KSM 22 DXAMase in mouthwash product A.
Control, no DXAMase added in mouthwash; Test, the addition of DXAMase in mouthwash. Detail reaction conditions are described in Materials and Methods.

Effect of *L. starkeyi* KSM 22 DXAMase in Mouthwash Product A on the Formation of Insoluble Glucan

The effect of DXAMase in mouthwash on the formation of insoluble glucan is shown in Fig. 3. It shows that the addition of mouthwash (containing DXAMase) into the glucosyltransferase reaction digest with sucrose inhibited insoluble glucan formation. Based on the turbidity comparison, an addition of DXAMase (0.1 IU/ml) reduced 80% of insoluble glucan formation compared to that of the control (no addition of DXAMase).

Effect of KSM 22 DXAMase in Mouthwash Product A on the Reduction of Pre-Formed Insoluble Glucan

DXAMase in mouthwash also removed the pre-formed films of glucan-bound *S. mutans* cells in glass vial.

DISCUSSION

Dextranase and amylase are enzymes which hydrolyze the α-1,6 or α-1,4-D-glucosidic linkages in sugar, which is present as a main constituent of dental plaque as a contaminant. Commercially, dextranases are produced by either *Penicillium* sp. or *Chaetomium* sp. Neither enzyme is US Food and Drug Administration approved, because of the difficulty of ensuring safety as a consequence of the sources of these enzymes. Both genera are known to produce antibiotics and toxins. *L. starkeyi* also produces a dextranase and/or amylase, and has been used in food-related applications. In addition, a mutant, *L. starkeyi* ATCC 74054, produces amylase with high yield [10, 11]. Therefore, the probability of FDA approval of dextranase and amylase produced by *L. starkeyi* is higher than that for other fungal sources. The objective of this study was to characterize the DXAMase (containing both dextranase and amylase activities) produced by *L. starkeyi* KSM 22, a mutant isolated from *L. starkeyi* ATCC 74054 for hyper-production of constitutive DXAMase, in respect to its application as agents for preventing dental plaque. DXAMase also effectively hydrolyzed insoluble glucan and produced glucose as a major product (Figs. 1 and 4). This indicates that the DXAMase has mutanase activity as well as both dextranase and amylase activities. This property is useful for dental plaque removal. We have examined the effect of general enzyme inhibitors that are also included in mouthwash or toothpaste as active ingredients. The DXAMase was stable with the fluoride (0.05%, w/v) and EDTA (0.29%, w/v) concentrations in toothpastes or mouthwash. Interestingly, the DXAMase was very stable even with 15-20% (v/v) ethanol. This means that this enzyme can be an additive to various antiseptics which usually contain about 20% ethanol. To confirm its possibility for use as an active ingredient in mouthwashes, we examined the effects of chemical ingredients of mouthwash on the activity and the stability of DXAMase. Sodium benzoate did not inhibit the DXAMase. In the case of cetylpyridinium chloride, although inactivation was concentration dependent, the DXAMase still showed almost 90% of initial activity at 0.05% (w/v) which is the concentration in most mouthwash products. The DXAMase activity was very stable at room temperature in commercial mouthwash products. The DXAMase added in mouthwash for longer than 6 months still showed effective inhibition of the insoluble glucan formation and removal of pre-formed insoluble glucan.

Water-insoluble glucans produced from sucrose are involved in the accumulation of *S. mutans* on tooth surfaces and are components of the dental plaque matrix. The glucans contain a relatively high proportion of α-1,3-, α-1,4-, and α-1,6-linked glucose residues. Consequently, they are highly resistant to hydrolysis by dextranase alone [6, 24]. Thus, the mutanolytic, amylolytic, and dextranolytic

activities are necessary for efficient removal of dental plaque [5, 8]. The DXAMase has all three activities (Figs. 1 and 4). A focus of recent research in preventive dentistry has been the search for easily administered chemical and biochemical agents as adjuncts to brushing and flossing for the removal of supragingival plaque and control of gingivitis [7, 17, 18]. Our *L. starkeyi* KSM 22 DXAMase containing mutanase, dextranase, and amylase activities is a potential as a dental plaque control agent. These enzymes can be easily and simultaneously produced in high yield by fermentation using starch with nominal cost.

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REFERENCES

1. Barrett, J. F., T. A. Barrett, and R. Curtiss. 1987. Purification and partial characterization of the multicomponent dextranase complex of *Streptococcus sobrinus* and the cloning of the dextranase gene. *Infect. Immun.* **55:** 729-802.
2. Bramstedt, F. and C. J. Lusty. 1968. The nature of the intracellular polysaccharides synthesized by *Streptococci* in the dental plaque. *Caries Res.* **2:** 201-213.
3. Charles, F. S., R. H. Staat, and S. K. Harlander. 1975. Dextranase from oral bacteria: Inhibition of water-insoluble glucan production and adherence to smooth surfaces by *Streptococcus mutans*. *Infect. Immun.* **12:** 309-317.
4. Fox, J. D. and J. F. Robyt. 1991. Miniaturization of three carbohydrate analyses using a microsample plate reader. *Anal. Biochem.* **195:** 93-96.
5. Guggenheim, B., B. Regorati, and H. R. Muhlenman. 1972. Caries and plaque inhibition by mutanase in rats. *Caries Res.* **6:** 289-297.
6. Hare, M. D., S. Svensson, and G. J. Walker. 1978. Characterization of the extracellular water-insoluble α-D-glucans of oral streptococci by methylation analysis and by enzymatic synthesis and degradation. *Carbohydr. Res.* **66:** 245-264.
7. Inoue, M., T. Yakushiji, M. Katsuki, N. Kudo, and T. Koga. 1988. Reduction of the adherence of *Streptococcus sobrinus* insoluble α-D-glucan by endo-(13)-α-D-glucanase. *Carbohydr. Res.* **182:** 277-286.
8. Jung, J. 1997. Isolation, identification, and characterization of mutanase from *Microbispora rosea*. Ph.D. Thesis, Chonnam National University, Korea.
9. Kim, D. and D. F. Day. 1994. A new process for the production of clinical dextran by mixed-culture fermentation of *Lipomyces starkeyi* and *Leuconostoc mesenteroides*. *Enzyme Microb. Technol.* **16:** 844-848.
10. Kim, D. and D. F. Day. 1995. Isolation of a dextranase constitutive mutant of *Lipomyces starkeyi* and its use for the production of clinical size dextran. *Lett. Appl. Microbiol.* **20:** 268-270.
11. Kim, D., H. C. Seo, and D. F. Day. 1996. Dextran production by *Leuconostoc mesenteroides* in the presence of a dextranase producing yeast, *Lipomyces starkeyi*. *Biotechnol. Tech.* **10:** 227-232.
12. Koenig, D. W. 1988. The dextranase of *Lipomyces starkeyi* and its use in sugar cane process. Ph.D. Thesis, Louisiana State University.
13. Koenig, D. W. and D. F. Day. 1989. Induction of *Lipomyces starkeyi* dextranase. *Appl. Environ. Microbiol.* **55:** 2079-2081.
14. Koga, T., S. Hamada, S. Murakawa, and A. Endo. 1982. Effect of a glucosyltransferase inhibitor on glucan synthesis and cellular adherence of *Streptococcus mutans*. *Infect. Immun.* **38:** 882-886.
15. Koga, T. and M. Inoue. 1978. Cellular adherence, glucosyltransferase adsorption, and glucan synthesis of *Streptococcus mutans* AHT mutants. *Infect. Immun.* **19:** 402-410.
16. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685.
17. Marsh, P. D. and D. J. Bradshaw. 1995. Dental plaque as a biofilm. *J. Ind. Microbiol.* **15:** 169-175.
18. Murayama, Y., H. Wada, H. Hayashi, T. Uchida, I. Yocomizo, and S. Hamada. 1973. Effects of dextranase from *Spicaria violaceae* (IFO 6120) on the polysaccharides produced by oral streptococci and on human dental plaque. *J. Dental Res.* **52:** 652-667.
19. Mukerjea R., D. Kim, and J. F. Robyt. 1996. Simplified and improved methylation analysis of saccharides, using a modified procedure and thin-layer chromatography. *Carbohydr. Res.* **292:** 11-20.
20. Mukasa, H. and H. D. Slade. 1974. Mechanism of adherence of *Streptococcus mutans* to smooth surfaces. II. Nature of the binding site and the adsorption of dextran-levan synthetase enzymes on the cell-wall surface of the *Streptococcus*. *Infect. Immun.* **9:** 419-429.
21. Phaff, H. J. and C. P. Kortzaman. 1984. Genus 14. *Lipomyces*, pp. 252-262. *In* R. Kreger-van. (ed.), *The Yeast A - Taxonomic Study*. Elsevier, Amsterdam.
22. Stoppelaar, J. D., J. Houte, and C. E. Moor. 1967. The presence of dextran-forming bacteria, resembling *Streptococcus bovis* and *Streptococcus sanguis*, in human dental plaque. *Arch. Oral Biol.* **12:** 1199-1202.
23. Takehara, T. and M. Inoue. 1981. Inhibitory effects of endo-α-1,3-glucanase on glucan film formation and glucan synthesis by the glucosyltransferase of the oral bacterium *Streptococcus mutans*. *Arch. Oral Biol.* **26:** 217-222.
24. Tsumuraya, Y. and A. Misaki. 1979. Structure of the water-insoluble alpha-D-glucan of *Streptococcus salivarius* HHT. *Carbohydr. Res.* **74:** 217-225.