Glycoconjugate Journal 16, 351–355 (1999)


# Processing α-glucosidase I is an inverting glycosidase

Monica M. Palcic[1]*, Christine H. Scaman[2], Albin Otter[1], Adam Szpacenko[1], Andrew Romaniouk[3], Ying Xia Li[2] and Inder K. Vijay[3]

[1]*Department of Chemistry, University of Alberta, Edmonton, Alberta, T6G 2G2 Canada*
[2]*Department of Food Science, University of British Columbia, 6650 Marine Drive, Vancouver, British Columbia, V6T 1Z4 Canada*
[3]*Department of Animal and Avian Sciences, University of Maryland, College Park, MD 20742 USA*

**α-Glucosidase I is a key enzyme in the biosynthesis of asparagine-linked oligosaccharides catalyzing the first processing event after the *en bloc* transfer of $Glc_3Man_9GlcNAc_2$ to proteins. This enzyme is an inhibitor target for anti-viral agents that interfere with the formation of essential glycoproteins required in viral assembly, secretion and infectivity. Of fundamental mechanistic interest for all oligosaccharide hydrolyzing enzymes is the stereochemical course of the reaction which can occur with either retention or inversion of anomeric configuration. The stereochemistry is used to categorize enzymes and is important in designing mechanism-based inhibitors. To determine the stereochemical course of the α-glucosidase I reaction, the release of glucose from a synthetic trisaccharide substrate, $Glc(\alpha1\text{-}2)Glc(\alpha1\text{-}3)Glc\alpha O(CH_2)_8COOCH_3$ was directly monitored by $^1H$ NMR spectroscopy. Both the yeast and bovine mammary gland enzymes released β-glucose concomitant with the formation of the $Glc(\alpha1\text{-}3)Glc\alpha O(CH_2)_8COOCH_3$ disaccharide product demonstrating that both enzymes operate with inversion of anomeric configuration.**

***Keywords:*** **stereochemistry, α-glucosidase I, $^1H$ NMR spectroscopy**

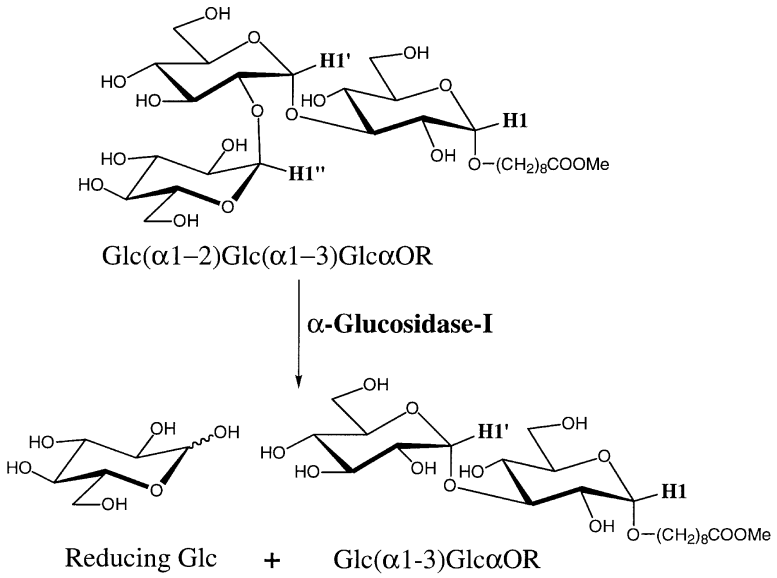
## Introduction

A key enzyme in the biosynthesis of asparagine-linked oligosaccharides is α-glucosidase I [1–3]. This enzyme catalyzes the hydrolysis of the terminal α1,2-linked glucose unit in $Glc_3Man_9GlcNAc_2$-structures after they are transferred *en bloc* to asparagine residues in an Asn-X-Ser/Thr motif [4]. Subsequent hydrolytic processing by α-glucosidase II and α-mannosidases and elaboration by glycosyltransferases results in the wide variety of glycosylation patterns found on glycoproteins. In the normal biosynthetic pathway of *N*-linked glycoproteins, glucosidase I is at a critical point in the regulation of glycoprotein biosynthesis since no further modification of *N*-linked oligosaccharides can occur until the terminal α1,2-glucose unit is removed. However, an alternate mechanism has been identified by which complex carbohydrates can be generated in the absence of glucosidase I [5]. Glucosidase I and II reactions play significant roles in protein folding by allowing the binding of glycoproteins to the endoplasmic reticulum chaperones calnexin and calreticulin [6]. There is considerable interest in developing inhibitors of *N*-linked oligosaccharide processing enzymes, including glucosidase I, as antiviral and antitumor agents [7,8]. However, no mechanistic or structural studies have been carried out on this enzyme in part due to difficulties in obtaining large quantities of the natural substrate and enzyme.

Glycoside hydrolyzing enzymes can be placed into two distinct categories, those that catalyze hydrolysis with net retention of anomeric configuration and those that hydrolyze with inversion of configuration [9–11]. Inverting glycosidase reactions are thought to occur by a direct displacement -mechanism while retaining glycosidases are believed to operate *via* a double-displacement mechanism with formation of a glycosyl-enzyme intermediate. The active site geometry of catalytic residues in the two classes is different and can be a factor in the design of enzyme inhibitors.

We have recently synthesized a truncated version of the $Glc_3Man_9GlcNAc_2$ structure as a specific substrate for glucosidase I (Scheme 1) [12,13] since simple *p*-nitrophenyl-glucosides are not hydrolyzed by this enzyme. This trisaccharide, $Glc(\alpha1\text{-}2)Glc(\alpha1\text{-}3)Glc\alpha O(CH_2)_8COOCH_3$, can be used to determine the stereochemical course of

*To whom correspondence should be addressed. Department of Chemistry, University of Alberta, Edmonton, Alberta, T6G 2G2 Canada. Tel: 780-492-0377; Fax: 780-492-7705; E-mail monica.palcic@ualberta.ca




**Scheme 1.** A synthetic trisaccharide substrate for glucosidase I

hydrolysis by directly monitoring the anomeric configuration of the released glucose by $^1$H NMR spectroscopy. In this study we establish that bovine mammary gland and yeast α-glucosidase I are both inverting hydrolases.

## Materials and methods

### Enzymes

Soluble yeast glucosidase I was isolated as previously described [12] except that a source Q column (0.5 × 5 cm) was utilized in place of a mono-Q HRS 5/5 column. Fractions containing glucosidase I that eluted from the column were pooled and exchanged into 50 mM deuterated sodium phosphate buffer, pD 7.1 (where pD is the uncorrected pH meter reading) using Centricon 10 units. Bovine enzyme was purified from lactating mammary glands essentially as described by Shailubhai et al., [14]. Microsomes prepared from 2 kg of fresh tissue were extracted four times with 10 mM sodium phosphate buffer, pH 6.8, containing 0.5% Triton X-100 to remove glucosidase II. The residual microsomal pellet was solubilized in 200 mM sodium phosphate buffer, pH 6.9, containing 0.8% Lubrol PX (buffer A). The Lubrol PX extract was bound to a N-carboxypentyl-1-deoxynojirimycin column (2 μmoles/g of swollen gel) [15] and eluted with buffer A containing 5 μM 1-deoxynojirimycin (DNJ). DNJ was removed from the enzyme by extensive dialysis against HPLC-grade water at 4 °C and enzyme was concentrated to near dryness on a lyophilizer. One ml of deuterated sodium phosphate buffer, pH 6.8, was added to the concentrated enzyme, which was exchanged into $D_2O$ buffer by dialysis three times against 25 ml of 25 mM sodium phosphate buffer, pD 6.8.

After exchange into deuterated buffer, the enzymes were assayed spectrophotometrically by monitoring glucose release from 2 mM Glc(α1-2)Glc(α1-3)GlcαO$(CH_2)_8$ $COOCH_3$ at 37 °C [12]. The activity of the yeast enzyme was 6.9 milliunits/ml and the bovine mammary gland enzyme 8.9 milliunits/ml where a milliunit is the amount of enzyme that converts 1 nmol of substrate to product per min. For NMR experiments, substrate and buffers were exchanged by lyophilization four times from $D_2O$.

### NMR

$^1$H-NMR spectra were acquired at 27.0 °C on a Varian Inova 600 spectrometer using VNMR 5.3B software. Chemical shifts are reported relative to 0.1% external acetone at 2.225 ppm. After initial acquisition of the reference enzyme spectra (700 μl in 5 mm NMR tubes), 60 μl of concentrated trisaccharide substrate were added to give a final concentration of 2.7 mM for the bovine enzyme and 9.3 mM for the yeast enzyme. A spectrum was recorded after 3 min, then every five min for 2.5 h and finally every 30 min until hydrolysis was complete. In this way the reaction's progress was monitored up to 15 h. Spectra were processed with a Gaussian window function of width *at*/4 and shifted by *at*/6 where *at* is the acquisition time. A first order baseline correction was applied prior to data analysis, integration and plotting. For integrations, the H1 signal that remains unchanged over the course of the reaction was used as a reference.

## Results and discussion

The stereochemical course of α-glucosidase reactions can be determined using $^1$H NMR spectroscopy to directly

monitor the release of glucose from the synthetic trisaccharide substrate Glc(α1-2)Glc(α1-3)GlcαO$(CH_2)_8COOCH_3$. The time course of hydrolysis catalyzed by the yeast enzyme is shown in Figure 1 where partial spectra of the anomeric region are shown. The signals corresponding to the anomeric protons of each of the glucose residues in the trisaccharide substrate (Scheme 1), H1″ at 5.54 ppm, H1′ at 5.18 ppm and H1 at 4.89 ppm and disaccharide product H1′ at 5.34 ppm are well resolved from each other, residual HDO at 4.75 ppm and from free glucose. Three min after the addition of enzyme, the anomeric proton of β-glucose is present at δ = 4.65 ppm, as well as disaccharide product H1′ signal at 5.34 ppm. In the case of the yeast enzyme, the β-glucose-H1 signal reaches the 5% level after 13 min (Fig. 2). This level is reached by the α-anomer-H1 after 53 min, indicating a lag time of ca. 40 min. The yeast α-glucosidase I reaction therefore occurs with inversion of anomeric configuration. After 5.5 hr the trisaccharide-H1″ signal as well as the trisaccharide-H1' signal disappear into the noise of the spectrum while the buildup of disaccharide-H1′ is complete. At this point the equilibrium of β:α glucose-H1 is 67:33 or 2:1 and does not change within experimental error. Of interest is the evolution of the trisaccharide/disaccharide H1 signal with time in Figure 1. The lines of the doublet at 4.91 ppm appear to get broader as the reaction progresses but as the experiment continues they become as sharp as before. This is due to a slight chemical shift change of the H1 resonance in going from the trisaccharide to the disaccharide. When both are present in an approximately equal ratio, the slightly different chemical shift and similar intensities lead to a broad signal. The change in chemical shift over the entire reaction is only +0.004 ppm (2.4 Hz) while the $^3J_{H1H2}$ coupling constant remains unchanged at 3.8 Hz.

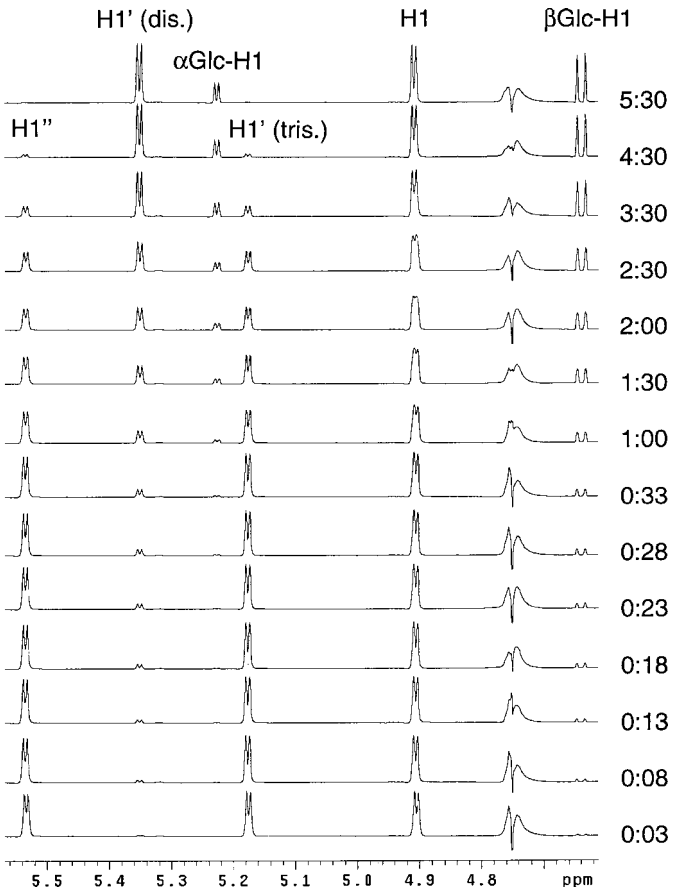



**Figure 1.** Proton spectra of the anomeric region of the trisaccharide Glc(α1-2)Glc(α1-3)GlcαOR at different times of incubation, indicated to the right of the spectra, with yeast α-glucosidase I. Anomeric peaks are labeled and the abbreviation dis. and tris. refer to the disaccharide and trisaccharide, respectively.

The progress of the reaction for the bovine mammary gland enzyme is shown in Figure 3. Although good spectral resolution, comparable to the yeast enzyme, was obtained at the beginning of the experiment (bottom trace), the resolution of all peaks and not just H1, deteriorated considerably with time. Therefore, after 2.5 h reaction time, the magnet was re-shimmed without interrupting the timing of the experiment, resulting in much sharper lines in the last six traces of Figure 3. While this greatly affects the appearance of the peaks, it has no effect on integrals that were used to analyze the progress of the reaction. The reaction is slower for the bovine enzyme since the substrate was present at a low sub-saturating concentration. The 5% level was reached after 33 min by the β-glucose-H1 signal and after 93 min by the α-glucose-H1 signal. Thus the lag time is of the order of one h. Due to the lower concentration of substrate and more baseline distortion by the protein, integrations are not as precise as in the yeast case. As with the yeast enzyme, the equilibrium concentration of β:α glucose-H1 is 67:33 or 2:1 after 14.5 h. Equilibrium is reached in both cases at completion of the reaction since the production of new β-glucose is very small and the time interval between measurements large (half hour and one hour, respectively) such that within experimental error the α:β ratio does not change. However, for both enzymes the measurement immediately before completion shows a ratio with slightly more β than α anomer (β:α 68:32) than at equilibrium. For both yeast and mammary gland enzyme it is clearly evident that the formation of α-glucose is delayed with respect to β-glucose, demonstrating that the hydrolytic reaction is an inverting process.

Recently, the processing α1,2-mannosidase in *Saccharomyces cerevisiae* was also shown to be an inverting enzyme [16]. Taken together with the results of this study on glucosidase I, it is tempting to speculate that more glycosidases for glycoprotein processing may be found to catalyze the hydrolysis of their substrates in the secretory path with inversion mechanisms.

A number of classification schemes have been proposed to categorize glycoside hydrolyzing enzymes on the basis of substrate specificity, mechanism of action and amino acid

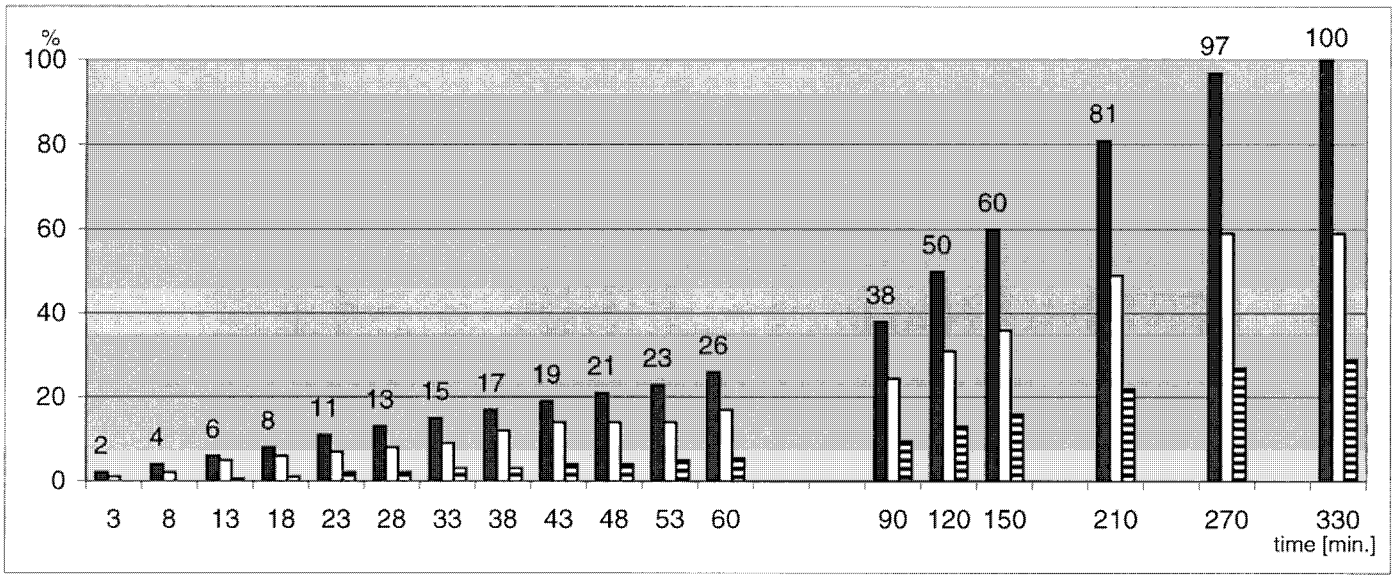



**Figure 2.** A plot of released α-Glc (dashed bars), β-Glc (white bars) and the total disaccharide formed (solid bars) as a function of time for the yeast α-glucosidase reaction. Total disaccharide percentage values are based on the integrated value of the H1′ anomeric proton. The reaction is complete after 5.5 hr. After 13 min the β-glucose-H1 signal is at the 5% level demonstrating that hydrolysis occurs with inversion of configuration. There is a lag of 40 min before the α-glucose-H1 signal reaches the 5% level as a result of the anomerization of the β-glucose released by the enzyme.

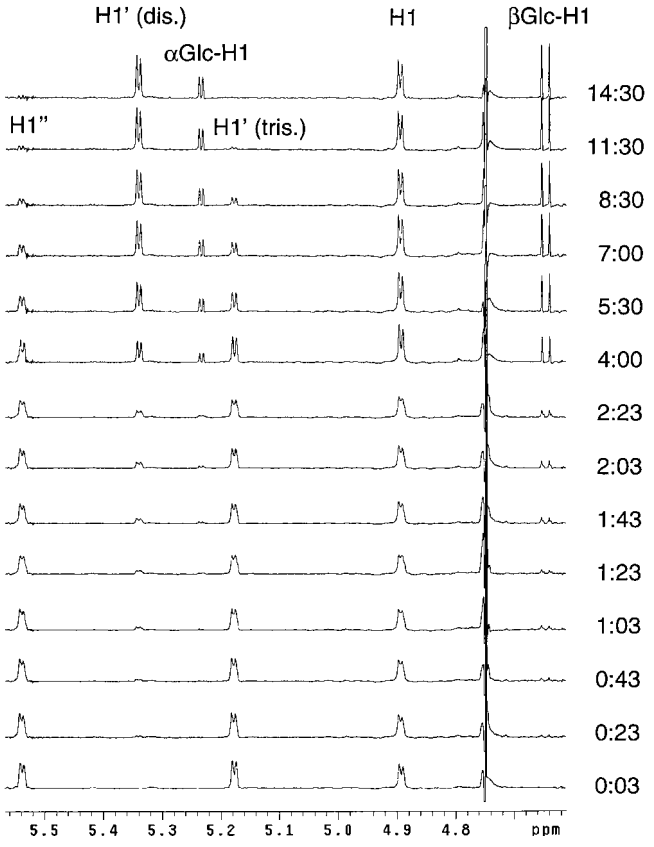



**Figure 3.** Proton spectra of the anomeric region of the trisaccharide Glc(α1-2)Glc(α1-3)GlcαOR at different incubation times, indicated to the right of the spectra, with bovine mammary gland α-glucosidase I. The reaction is complete after 14.5 hr. For further details see the legend to Figure 1 and the text.

sequence similarities [17]. To date the human hippocampus and yeast α-glucosidase I enzyme have been sequenced [18,19], and there is no structural information for any glucosidase I enzyme. A sequence-based classification has identified 66 different families of glycoside hydrolases [17,20], although reclassification may occur once further structural and mechanistic information becomes available. The human hippocampus and yeast enzymes presently comprise a distinct family 63 with no homology to other families.

Glycoside hydrolases generally contain two catalytic groups, usually aspartate or glutamate residues in their active sites. Inverting reactions are thought to occur with the base-catalyzed attack of water on the anomeric center along with the acid-catalyzed cleavage of the glycosidic bond, resulting in the direct displacement of the substrate leaving group by water. In this mechanism, one residue acts as a general base by abstracting a proton from the incoming water molecule, while the second acts as a general acid and protonates the leaving group [10,11]. In mechanisms for retaining enzymes, one of the two acidic amino acid residues is believed to act as a nucleophile, attacking the anomeric carbon and generating a glycosyl-enzyme intermediate or oxocarbenium-ion like intermediate after departure of the leaving group. The second residue functions as a general acid/base catalyst. X-ray crystallographic studies of over twenty glycosidases reveal the distance between these two catalytic groups is generally 4.8–5.5 Å in retaining enzymes and 9–10 Å in inverting enzymes [21,22]. The longer distance between these residues in inverting en-

zymes is presumably required to accommodate the catalytic water molecule. Therefore, establishing that the stereochemical course of the reaction of glucosidase I occurs with inversion of configuration, coupled with other mechanistic and structural data, such as the identity of the catalytic residues, should prove useful in the rational design of inhibitors of this enzyme as has been done for retaining glycosidases using 2-deoxy-2-fluoro glycosides as mechanism based inhibitors [23].

## Acknowledgments

This work was supported by funding from the Natural Sciences and Engineering Research Council of Canada (OGP 3045 to M.M.P. and OGP183895 to C.H.S.), N.I.H (DK 19682 to I.K.V.) and the Maryland Agricultural Experiment Station (Project C-118) and a Distinguished Faculty Research Fellowship (to I.K.V.).

## References

1 Kilker RD Jr, Saunier B, Tkacz JS, Herscovics A (1981) *J Biol Chem* **256:** 5299–303.
2 Hettkamp H, Legler G, Bause E (1984) *Eur J Biochem* **142:** 85–90.
3 Moremen KW, Trimble RB, Herscovics A (1994) *Glycobiology* **4:** 113–25.
4 Kornfeld R, Kornfeld S (1985) *Ann Rev Biochem* **54:** 631–64.
5 Moore SE, Spiro RG (1992) *J Biol Chem* **267:** 8443–51.
6 Helenius A (1994) *Mol Biol Cell* **5:** 253–65.
7 Elbein AD (1991) *FASEB J* **5:** 3055–63.
8 Mehta A, Rudd PM, Block TM, Dwek RA (1997)*Biochem Soc Trans* **25:** 1188–93.
9 Koshland DE (1953) *Biol Rev* **28:** 416–36.
10 Legler G (1990) *Adv Cabohydr Chem Biochem* **48:** 319–84.
11 Sinnot ML (1990) *Chem Rev* **90:** 1171–202.
12 Neverova I, Scaman CH, Srivastava OP, Szweda R, Vijay IK, Palcic MM (1994) *Anal Biochem* **222:** 190–95.
13 Scaman CH, Hindsgaul O, Palcic MM, Srivastava, OP (1996) *Carbohydr Res* **296:** 203–13.
14 Shailubhai K, Pratta, MA, Vijay, IK (1987) *Biochem J* **247:** 555–62.
15 Shailubhai K, Pukazhenthi B, Saxena ES, Varma G, Vijay IK (1991) *J Biol Chem.* **266:** 16587–93.
16 Lipari F, Gour-Salin BJ, Herscovics A (1995) *Biochem Biophys Res Commun* **209:** 322–26.
17 Henrissat B, Davies G (1997)*Curr Opin Struct Biol* **7:** 637–44.
18 Kalz-Füller B, Bieberich E, Bause E (1995) *Eur J Biochem* **231:** 344–51.
19 Romero PA, Dijkgraaf GJP, Shahinian S, Herscovics A, Bussey, H (1997) *Glycobiology* **7:** 997–1004.
20 Henrissat B, Bairoch A (1996) *Biochem J* **316:** 695–96.
21 McCarther JD, Withers SG (1994) *Curr Opin Struct Biol* **4:** 885–92.
22 Davies G, Henrissat B (1995) *Structure* **3:** 853–59.
23 Withers SG, Aebersold R (1995) *Protein Sci* **4:** 361–72.

Received 23 March 1999, revised 8 June 1999, accepted 12 June 1999