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Enantioselective microbial reduction of 6-oxo-8-[4-[4-(2-pyrimidinyl)-1-piperazinyl]butyl]-8-azaspiro[4.5]decane-7,9-dione

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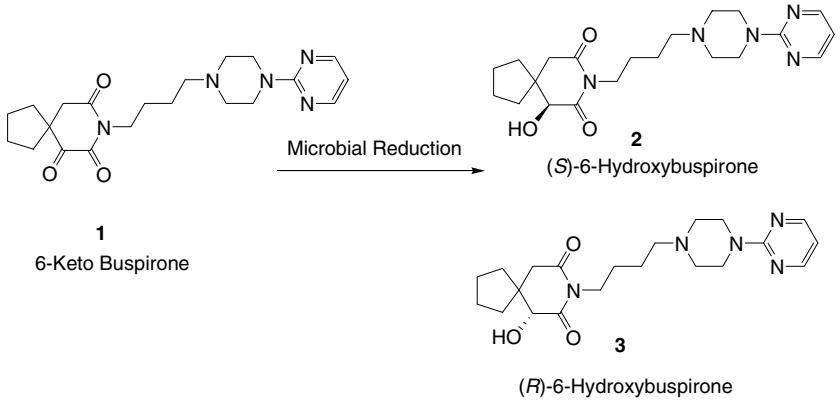
Abstract—The enantioselective microbial reduction of 6-oxo-8-[4-[4-(2-pyrimidinyl)-1-piperazinyl]butyl]-8-azaspiro[4.5]decane-7,9-dione **1** to either of the corresponding (*R*)- or (*S*)-6-hydroxy-8-[4-[4-(2-pyrimidinyl)-1-piperazinyl]butyl]-8-azaspiro[4.5]decane-7,9-diones **2** and **3** is described.

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is involved in various physiological and pathological processes by its interaction with seven classes of receptors (5-HT_{1-7}) containing 14 distinct receptors grouped on the basis of amino acid sequence, pharmacology, and signal transduction pathways.[1,2] The 5-HT_{1A} receptor subtype has been the target of considerable research because of its involvement in psychiatric disorders, such as anxiety and depression.[3,4] Earlier studies focused on the development of $5HT_{1A}$ receptor agonists, such as buspirone, the first $5HT_{1A}$ receptor agent launched in the market. More recently, new therapeutic perspectives have been proposed: $5HT_{1A}$ receptor agonists may be useful as antidepressants[5,6] and as neuroprotective agents.[7] Although a large number of compounds with a high affinity for $5HT_{1A}$ receptors have been described in the past, only a few of them are both selective and highly efficacious at the receptor.[8–10] The metabolism of orally administered buspirone has been studied in rats.[11] The primary routes of metabolism involve hydroxylation at C-6′, C-3′, and C-5 to give 6′-hydroxybuspirone, 3′-hydroxybuspirone, and 5-hydroxybuspirone.[11,12] One of these metabolites, 6-hydroxy-8-[4-[4-(2-pyrimidinyl)-piperazinyl]-butyl]-8-azaspiro[4.5]-7,9-dione, and its



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pharmaceutically acceptable salts and hydrates were found to be useful in the alleviation of anxiety.[13]

Herein, we report the enantioselective microbial reduction of 6-oxo-8-[4-[4-(2-pyrimidinyl)-1-piperazinyl]-butyl]-8-azaspiro[4.5]decane-7,9-dione **1** to either of the corresponding (*R*)- or (*S*)-6-hydroxy-8-[4-[4-(2-pyrimidinyl)-1-piperazinyl]butyl]-8-azaspiro[4.5]decane-7,9-diones **2** and **3**.

2. Results and discussion

A number of oxidoreductases, including those from yeast,[14–18] horse liver,[19] *Thermoanerobic brockii*,[20,21] *Lactobacillus kefir*,[22] *Pseudomonas* sp.,[23] *Geotrichum candidum*,[24,25] *Hansenula polymorpha*,[26,27] *Mortierella rammaniana*,[28] and *Sulfolobus solfataricus*,[29] have proven useful for the enantioselective reduction of ketones to alcohols.

About 150 microorganisms were screened for the enantioselective reduction of **1**. The reaction yields and enantiomeric purity of (*S*)-6-hydroxybuspirone **2** obtained with the fifteen best cultures are shown in Table 1; lower ees (<80%) and reaction yields (<37%) were obtained with other cultures. *Rhizopus stolonifer* SC 13898, SC 16199, *Neurospora crassa* SC 13816, *Mucor racemosus* SC 16198, *Rhizopus oligosporus* SC 16197, *Pseudomonas putida* SC 13817, SC 16030, SC 16260, *Rhodococcus* sp. SC 13962, *Rhodotorula mucilaginosa* SC 16357, and *Trichoderma virdiae* SC 13826 gave >44% reaction yields and >81% ees of (*S*)-6-hydroxybuspirone **2**.

Table 1. Enantioselective microbial reduction of ketone **1** to (*S*)-6-hydroxybuspirone **2**

Microorganisms	Yield of **2** (%)	ee of **2** (%)
Rhizopus stolonifer SC 16199	54	95.4
Rhizopus stolonifer SC 13898	53	96.6
Neurospora crassa SC 13816	59	95.6
Mucor Racemosus SC 16198	44	95.5
Rhizopus oligosporus SC 16197	61	95.4
Mucor plumbus SC 13993	44	94.2
Mucor circinelloides SC 13976	47	93.5
Mucor rouxii SC 13920	37	91.2
Mucor hiemalis SC 13826	47	91
Pseudomonas putida SC 16030	70	89
Pseudomonas putida SC 13868	63	83
Pseudomonas putida SC 16269	75	82
Rhodococcus sp. SC 13962	51	87
Rhodotorula mucilaginosa SC 16357	50	81
Trichoderma viridae SC 13826	63	84

Among the cultures evaluated, the yeast strains *Candida*, *Hansenula*, *Pichia*, *Kloeckera*, and some bacterial cultures, *Brevibacterium linens* SC 13959, *Mycobacterium* sp. SC 13900, *Providencia alcalifaciens* SC 9036, and *Pseudomonas cepacia* SC 13867, gave (*R*)-6-hydroxybuspirone **3** in >90% enantiomeric excess (Table 2).

Table 2. Enantioselective microbial reduction of ketone **1** to (*R*)-6-hydroxybuspirone **3**

Microorganisms	Yield of **3** (%)	ee of **3** (%)
Candida maltosa SC 16112	57	97.7
Candida parapsilosis SC 16348	52	96
Brevibacterium linens SC 13959	57	92
Hansenula polymorpha SC 13824	12	93
Hansenula polymorpha SC 13845	25	98
Kloeckera sp. SC 13823	36	95
Pichia methanolica SC 13825	26	96
Lodderomyces elongisporus SC 16110	34	93
Mycobacterium sp. SC 13900	20	89
Pichia methanolica SC 16415	59	90
Pichia pinus SC 13864	53	82
Providencia alcalifaciens SC 9036	34	90
Pseudomonas cepacia SC 13867	51	80

H. polymorpha SC 13845 and *Candida maltosa* SC 16112 were investigated further for conversion of ketone **1** to (*R*)-6-hydroxybuspirone **3**, and *R. stolonifer* SC 16002 and *P. putida* 16269 were used to convert ketone **1** to (*S*)-6-hydroxybuspirone **2**. Cells of *H. polymorpha* SC 13845 and *C. maltosa* SC 16112 were grown in a 25-L fermentor for 48 h, and then collected and suspended in 2 L of 80 mM potassium phosphate buffer (pH 7.0) at 20% (w/v wet cells) concentration and the resulting cell suspensions were used to carry out the two-stage process for biotransformation of **1** as described in the Experimental section. After 48 h, reaction yields of >62% and ees of >97% were obtained for alcohol **3** using each culture (Table 3). From a 545-mL reaction mixture derived from *H. polymorpha* SC 13845, 3.44 g of **3** was isolated.

Cell extracts of *H. polymorpha* SC 13845 were then evaluated for the reduction of ketone **1**. Glucose dehydrogenase in the presence of glucose and NADP or formate dehydrogenase in the presence of formate and NAD were used to regenerate the cofactor (NADPH or NADH, respectively) required for the reduction. Higher reaction yields and ees were obtained for chiral alcohol **3** (52% yield, 98% ee) when NADPH was used as a cofactor compared to NADH (Table 4).

The NADP-dependent dehydrogenase that catalyzes the reduction of **1** to (*R*)-6-hydroxybuspirone was purified 125-fold to homogeneity from cell extracts of

Table 3. Enantioselective microbial reduction of ketone **1** to (*R*)-6-hydroxybuspirone **3** (two-stage process)

Microorganisms	Reaction time (h)	Substrate input in 1 L reactor (g)	Yield of **3** (%)	ee of **3** (%)
Hansenula polymorpha SC 13845	20	10	40	98
	48		65	
Candida maltosa SC 16112	20	10	38	97
	48		62	

Table 4. Enantioselective microbial reduction of ketone **1** to (*R*)-6-hydroxybuspirone **3**: cell-free extracts of *Hansenula polymorpha* SC 13845

Regeneration system	Reaction time (h)	Substrate input in 10 mL reactor (mg)	Yield of **3** (%)	ee of **3** (%)
NAD, Formate and formate dehydrogenase	6	10	20	90
NADP, Glucose and glucose dehydrogenase	6	10	52	98

H. polymorpha SC 13845 (Table 5). The sub-unit molecular weight of the enzyme is 35,000 Da based on SDS/PAGE. The molecular weight of the enzyme as determined by gel filtration (Sephacryl S-200) column chromatography is about 36,000 indicating that the enzyme is monomeric.

Table 5. Purification of dehydrogenase from *Hansenula polymorpha* SC 13895

Steps	Volume (mL)	Total protein (mg)	Total units	Specific activity (units/mg)
Cell extract	45	123	22.6	0.18
Blue-dye affinity	10	6.8	12.9	1.9
Sephacryl S-200	16	1.7	6.6	3.88
Sephadex S-75	2	0.3	2.8	9.33

Cells of *R. stolonifer* SC 13898 were grown in a 1-L fermentor for 24 h with continuous feeding of 100 mL of 10× concentrated medium during growth as described in the Experimental section. Substrate **1** was added gradually to the 1.1 L broth to a final input of 10 g/L. After 48 h, a reaction yield of 70% and ee of 99% were obtained for the (*S*)-6-hydroxybuspirone **2**. From a 900-mL reaction mixture, 4.74 g of **2** was isolated with an ee of 99.2% in 58% yield.

Cells of *P. putida* SC 16269 were grown in a 25-L fermentor for 48 h. The cells were collected and suspended in 2 L of an 80 mM potassium phosphate buffer (pH 7.0) at 20% (w/v wet cells) concentration and the resulting cell suspensions used to carry out the two-stage process for biotransformation of **1**. After 48 h, a reaction yield of 74% and ee of 82% were obtained for the alcohol **2**. Cell extracts of *P. putida* SC 16269 were then evaluated for reduction of ketone **1**. Glucose dehydrogenase in the presence of glucose and either NADP or formate dehydrogenase in the presence of formate and NAD were used to regenerate the cofactor (NADPH or NADH, respectively) required for the reduction. Higher reaction yields and ees were obtained for chiral alcohol **2** when NADH was used as a cofactor compared to NADPH. The results are shown in Table 6.

The oxidoreductases from yeast,[14–18] horse liver,[19] and *T. brockii*[20,21] transfer the pro-*R* hydride to the *re*-face of the carbonyl to give (*S*)-alcohols, a process described by Prelog's rule.[30] In contrast, oxidoreductases from *L. kefir*[22] and two *Pseudomonas* sp.[23] exhibit anti-Prelog specificity, transferring the pro-*R* hydride to form (*R*)-alcohols.

3. Conclusion

Herein, we have described the reduction of ketone **1** to (*S*)-6-hydroxybuspirone by fungal and bacterial cultures *R. stolonifer* SC 13898 and *P. putida* SC 16269, respectively. NADH is a better cofactor when compared to NADPH giving higher reaction yields and ee of the product **2** when cell extracts of *P. putida* SC 16269 were used for this reduction process. In contrast, the reduction of **1** by yeast cultures *H. polymorpha* SC 13845 and *C. maltosa* SC 16112 gave (*R*)-6-hydroxybuspirone **3**. NADPH is a better cofactor when compared to NADH, giving higher reaction yields and ee of **3** when cell extracts of *H. polymorpha* SC 13845 were used for this reduction.

4. Experimental

4.1. Materials

The starting substrate **1** and reference compounds **2** and **3** were synthesized by colleagues in Process Research and Development, Bristol-Myers Squibb Pharmaceutical Research Institute. The spectral characteristics (^{1}H NMR, ^{13}C NMR, mass spectra) of products **2** and **3** from enzymatic conversions were in full accordance with the corresponding synthetic materials. The proton magnetic resonance (^{1}HNMR) and carbon magnetic resonance (^{13}C NMR) spectra were recorded on a Brucker AM-300 spectrometer.

4.2. Microorganisms

Microorganisms (Tables 1 and 2) were obtained from the culture collection of the Bristol-Myers Squibb Pharmaceutical Research Institute. Microbial cultures were stored at −90 °C in vials.

4.3. Growth of microorganisms

For screening purposes, one vial of each culture was used to inoculate 100 mL of medium A containing 1% malt extract, 1% yeast extract, 2% glucose, and 0.3% peptone. The medium was adjusted to pH 6.8 before

Table 6. Enantioselective microbial reduction of ketone **1** to (*S*)-6-hydroxybuspirone **2**: cell-free extracts of *Pseudomonas putida* SC 16269

Regeneration system	Reaction time (h)	Substrate input in 10 mL reactor (mg)	Yield of **2** (%)	ee of **2** (%)
NAD, Formate and formate dehydrogenase	6	10	73	99
NADP, Glucose and glucose dehydrogenase	6	10	35	70

sterilization. Cultures were grown at 28 °C and 280 rpm for 48 h. Cultures were harvested by centrifugation at 18,000*g* for 15 min, washed with 50 mM pH 7.0 potassium phosphate buffer, and used for reduction studies.

4.4. Reduction of 1 by cell-suspensions

Cells of various microorganisms were suspended separately in 10 mL of 50 mM potassium phosphate buffer (pH 6.5) at 20% (w/v, wet cells) cell concentration and supplemented with 1 mg/mL of **1** and 30 mg/mL of glucose. Reduction was conducted at 28 °C and 150 rpm. Periodically, samples of 1 mL were taken and mixed with 1 mL acetonitrile, filtered through a 0.2 μm LID/X filter and analyzed by HPLC for determination of substrate and product concentration. To determine the enantiomeric excess of the product, a 2 mL sample was extracted with 8 mL of ethyl acetate; the separated organic layer was evaporated under a stream of nitrogen at 40 °C until dryness. The residue was dissolved in 1 mL of methanol and analyzed by chiral HPLC (see below).

4.5. Two-stage process for reduction of 1

R. stolonifer SC 13898, *P. putida* SC 16269, *H. polymorpha* SC 13845, and *C. maltosa* SC 16112 cultures were grown in a 25-L fermentor containing 15 L of medium A containing 0.025% UCON antifoam. Growth consisted of several inoculum development stages and fermentation. Inoculum development consisted of F1 and F2 stages. In the F1 stage, a frozen vial of each culture was inoculated into 100 mL of medium A contained in a 500-mL flask. Growth was carried out at 28 °C and 280 rpm for 48 h on a rotary shaker. In the F2 stage, 10 mL of the F1 stage culture was inoculated into 1 L of medium A and incubated at 28 °C and 280 rpm for 24 h.

Fermentors containing 15 L of medium A were inoculated with 1 L of inoculum of each culture from an F2 stage. Fermentation was conducted at 25 °C and 500 rpm with 15 Lpm (liter per min) aeration for 36 h. After 48 h fermentation, the cells were collected and stored at −90 °C until further use.

Frozen cells from the above batches were used to conduct the reduction of **1** in a 3-L reactor. Cell suspensions (20% w/v, wet cells) in 1 L of 70 mM potassium phosphate buffer (pH 7.0) were used. Compound **1** (10 g) and glucose (30 g) were added to the reactor and the reduction carried out at 28 °C and 160 rpm with 1 Lpm aeration for 49 h. The pH was maintained between 6.8 and 7.0. Periodically, samples were prepared as described above and analyzed by HPLC to determine the % conversion of **1**. The enantiomeric purity of the product was determined by chiral HPLC.

4.6. Single-stage process for the reduction of 1

R. stolonifer SC 13898 and *H. polymorpha* SC 13845 cultures were grown in a 5-L fermentor containing 2 L of medium A as described above. After 18 h of growth, 20 g of ketone **1** was added to the fermentor and the biotransformation process continued for 48 h. The pH was maintained at 5.5 during the biotransformation. Periodically, samples were prepared as described above and analyzed by HPLC to determine the conversion of **1**. The enantiomeric purity of the product was determined by chiral HPLC.

4.7. Preparation of ketobuspirone 1

A 500-mL, three-necked, round-bottomed flask equipped with an overhead stirrer and a temperature probe was placed under a nitrogen atmosphere. To this flask was charged ~130 mL of methylene chloride. Oxalyl chloride (5.4 mL, 62.2 mmol, 2.0 equiv) was added over 5 min. The contents of the flask were cooled to a temperature of ~−75 °C. Dimethyl sulfoxide (DMSO) (8.8 mL, 124 mmol, 4.0 equiv) was added slowly (dropwise) to the contents of the flask. The addition was exothermic and the temperature rose above −65 °C. The contents were stirred at −75 °C for another 15 min to complete the formation of the complex. Separately, a solution of 6-hydroxybuspirone (12.5 g, 31.1 mmol, 1.0 equiv) in methylene chloride (100 mL) was prepared. The methylene chloride solution of 6-hydroxybuspirone was added dropwise over a period of 15 min. The temperature rose to about −65 °C. The reaction mixture was stirred for another hour at −70 °C. Triethylamine (21.7 mL, 155.7 mmol, 5.0 equiv) was added over 15 min and the reaction mixture stirred at −70 °C for 20 min. The cooling bath was removed and the reaction mixture warmed to ambient temperature over a 2 h period. The slurry was filtered to remove the triethylamine hydrochloride formed in the reaction. The solution was filtered through a pad of silica gel and the fractions analyzed by TLC (using 5% methanol-methylene chloride).

Fractions containing the desired product were combined and concentrated at <30 °C to give a yellow solid. Ethyl acetate (40 mL) was added and the mixture stirred at ambient temperature. Additional triethylamine hydrochloride was filtered off and the solid washed with ~10 mL of ethyl acetate. The yellow filtrate was stored at room temperature overnight. Heptanes (40 mL) were added dropwise until cloudiness was observed; the mixture was then stirred for 20 min. An additional 150 mL of heptanes was added and the mixture cooled to −10 °C and held overnight. The slurry was filtered through a Buchner funnel. The flask was washed with ~30 mL of 1:6 ethyl acetate/heptanes and the wet cake was washed with an additional 30 mL of 1:6 ethyl acetate/heptanes. The product was dried in the funnel with vacuum for 16 h to give 7.4 g of a pale yellow solid for a yield of 60 M%. CNMR ($CDCl_3$, 75 MHz): δ 191.57, 169.85, 161.94, 159.56, 158.11, 110.24, 57.82, 53.43, 53.24, 43.92, 43.74, 42.82, 42.21, 34.69, 25.81 ppm. HNMR ($CDCl_3$, 300 MHz): δ 8.38 (2H, pyrimidine ring), 6.58 (^{1}H, pyrimidine ring), 3.48, (m, 2H), 3.16 (m, 4H, methylene on piperazine ring), 2.59 (m, 4H), 2.43 (2H), 2.36 (m, 2H), 1.76 (m, 4H), 1.53 (m, 6H), 1.39 (m, 2H) ppm. IR: 2945, 2858, 2827, 1726, 1675

(two different carbonyl groups), 1588, 1552, 1485, 1454, 1357, 1270, 1137, 1101, 988 cm^{-1}.

4.8. Isolation of 2 and 3

The *R. stolonifer* SC13898 reduction mixture (900 mL, pH 4.8, 6-oxobuspirone input: 8 g) was filtered and the filtrate extracted at pH 8 (K_3PO_4) with ethyl acetate. The extract was washed with water and concentrated in vacuo to give 4.74 g of (*S*)-6-hydroxybuspirone **2** with a potency of 97.4% and an ee of 99.4%.

The *Hansenula polymorha* SC13895 reduction mixture (545 mL, pH 3.4, 6-oxobuspirone input: 6 g) was filtered and the filtrate extracted at pH 7 (K_3PO_4) with ethyl acetate. The extract was washed with water and concentrated in vacuo to give 3.44 g of (*R*)-6-hydroxybuspirone **3** with a potency of 95.5% and an ee of 91.7%. NMR spectra were acquired in acetonitrile-d_3 with a Brucker Model DPX 300 spectrometer. Proton and carbon NMR spectra of the isolated (*S*)- and (*R*)-6-hydroxybuspirone were compared with the spectra of authentic racemic 6-hydroxybuspirone and were indistinguishable. The proton NMR data was in agreement with the data reported earlier.[31]

4.9. Analytical methods

Analysis of **1**, **2**, and **3** were carried out using a Hewlett Packard HPLC. A YMC Basic column (200 × 4.6 mm, ID 5 μ) was used under the following conditions. The mobile phase consisted of four different solvents. Solvent A contained 40% methanol with 60% 10 mM ammonium acetate, solvent B contained 80% methanol with 20% 10 mM ammonium acetate, solvent C contained 100% methanol, and solvent D contained 50% methanol with 50% water. The column temperature was 30 °C, the flow rate 1 mL/min, and the detection wavelength 238 nm. Run time was 25 min. The following gradient was used.

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)	Solvent D (%)
0.1	100	0	0	0
3.0	100	0	0	0
23.0	0	100	0	0
26.0	0	100	0	0
27.0	100	0	0	0
35.0	100	0	0	0
36.0	0	0	0	100
56.0	0	0	100	0
66.0	0	0	100	0

The retention times for substrate **1** and the corresponding racemic alcohol are 17.1 and 14.5 min, respectively. The enantiomeric excess of chiral alcohols **2** and **3** was determined by chiral HPLC. An Astec Chirobiotic column (100 × 4.5 mm, ID 5 μ) was used at ambient temperature; the injection volume was 10 μL; the mobile phase was 0.8% acetic acid and 0.4% triethylamine in methanol; the flow rate was 0.6 mL/min; and the detection wavelength was 243 nm. Retention times for compounds **2** and **3** were 7.9 and 9.4 min, respectively.

4.10. Preparation of cell extracts and reduction of 1 using cell extracts

The preparations of cell extracts were carried out at 4–7 °C. *H. polymorpha* SC 13895 and *P. putida* SC 16269 cells were washed with 50 mM potassium phosphate buffer (pH 6.1). The washed cells (30 g) were suspended in 200 mL of buffer containing 10% glycerol, 1 mM ethylenediamine tetraacetic acid (EDTA), and 1 mM dithiothreitol (DTT). Cell suspensions (10% w/v, wet cells) were disintegrated with a Microfluidizer (Microfluidics, Inc.) at 12,000 psi (two passages), and the disintegrated cells centrifuged at 25,000*g* for 30 min to obtain the cell extract. Protein in cell extracts was estimated using Bio-Rad protein reagent with bovine serum albumin as the standard. The assay mixture contained 1–10 μL of enzyme fraction, 0.8 mL water, and 0.2 mL Bio-Rad reagent. After mixing, the absorbance of the solution was measured at 595 nm. Cell extracts were evaluated for the biotransformation of ketone **1**. The reaction mixture contained 1 mg/mL substrate, 2 mM nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP), 8 units glucose dehydrogenase, and 2 mg/mL glucose or 1 mM formate and 3 units formate dehydrogenase to regenerate reduced cofactor (NADH or NADPH) required for the reduction reaction. Samples were incubated at 28 °C, 150 RPM. Concentrations of substrate and products were estimated by HPLC.

4.11. Purification of reductase from *H. polymorpha* SC 13895

H. polymorha cells were suspended at 10% (w/v, wet cells) in 500 mL of buffer A (10 mM phosphate buffer, pH 6.1 containing 10% glycerol, 1 mM DTT, 1 mM EDTA). The cell suspension was passed twice through a microfluidizer at 12,000 psi. The disintegrated cells were centrifuged at 25,000*g* for 15 min at 4 °C to obtain the cell extracts. The cell extract was loaded onto a 10-mL column (two Pharmacia 5-mL bluegel affinity columns connected in a row) at a flow rate of 1 mL/min. The column was washed with 40 mL of buffer A. The column was eluted with buffer A containing a gradient of NADP increasing from 0 to 10 mM. The reductase activity was eluted in fractions containing 0.25–0.5 mM NADP. Fractions containing activity were pooled and concentrated with an Amicon 10K Centriprep to 1 mL. The concentrated enzyme fraction was loaded onto a 84 × 2.5 cm Sephacryl S-200 column. Fractions of 8 mL were collected. Fractions containing enzyme activity (fractions 33–35) were pooled and concentrated by an Amicon 10K Centricon to 600 μL. The concentrated fraction was loaded onto a S75 Sephadex (FPLC) column and 1-mL fractions were collected. The enzyme activity was present in fractions 13 and 14. Both fractions were analyzed by 12.5% SDS-PAGE.

4.12. Sodium dodecyl sulfate polyacrylamide gel-electrophoresis (SDS/PAGE)

The active fractions from the Sephadex S75 column were evaluated by SDS-PAGE as described in the Phast-System® procedure by Pharmacia using the homogeneous 12.5% Phastgel. The enzyme samples were added to a buffer containing 10 mM Tris–HCl, 1 mM EDTA, pH 8, 2.5% SDS, and 5% β-mercaptoethanol. The mixture was heated at 100 °C for 5 min, and 0.01% bromophenol blue then added. Gels were stained with silver stain and destained in 10% acetic acid solution. Markers with standard molecular weights were phosphorylase β (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400).

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